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HIGH TEMPERATURE STRESS TOLERANCE IN WHEAT GENOTYPES: ROLE OF ANTIOXIDANT DEFENCE ENZYMES

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Received: 28 September, 2007; accepted: 26 December, 2008

Two wheat genotypes, C 306 (tolerant) and PBW 343 (susceptible to temperature stress) were grown in growth chambers in the phytotron facility of IARI, New Delhi. The plants were maintained at 18/23°C (control) and 25/35°C (temperature stress) night/day temperatures after maximum tillering. Water potential was significantly reduced at anthesis, and at 7 and 15 days after anthesis in both genotypes in the heat stress treatment, and a greater reduction was recorded in PBW 343. The membrane stability index was also lower in the heat stress treatment in both genotypes at the vegetative stage, at anthesis and at 15 days after anthesis, and a greater reduction was observed in PBW 343 than in C 306. The hydrogen peroxide content increased as the plants advanced in age, and a higher hydrogen peroxide content was recorded in PBW 343 than in C 306 at different stages of growth in the heat stress treatment. The superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) and peroxidase (POX) activities increased significantly at all stages of growth in C 306 in response to heat stress treatment, while PBW 343 showed a significant reduction in catalase, glutathione reductase and peroxidase activities in the high temperature treatment. Northern blot showed a significant increase in the *APX*-mRNA level under heat stress at the vegetative and anthesis stages, and the expression was greater in C 306. From the results it is apparent that the antioxidant defence mechanism plays an important role in the heat stress tolerance of wheat genotypes.

Key words: ascorbate peroxidase, catalase, gene expression, glutathione reductase, hydrogen peroxide, membrane stability index, peroxidase, superoxide dismutase, temperature stress

Introduction

High temperature limits the productivity of crops in many regions of the world (Al-Khatib and Paulsen, 1999) and continual heat stress is a problem on 7 million hectares, while terminal heat stress can be a problem on 40% of the irrigated wheat growing areas of the world (Fisher and Byerlee, 1990). Oxidative stress induced by high temperature has been reported in various higher and

lower plants (Upadhyaya et al., 1990; Jagtap and Bhargava, 1995; Davidson et al., 1996; Sairam et al., 1997; 2000). Heat injury in cool season grasses and *Arabidopsis* has been associated with oxidative damage (Jiang and Huang, 2001; Liu and Huang, 2000; Larkindale and Knight, 2002).

Tolerance to high temperature stress in crop plants has been associated with an increase in antioxidant enzyme activity (Rui et al., 1990; Gupta et al., 1993; Badiani et al., 1994; Zhau et al., 1995; Sairam et al., 1997; 2000; Chaitanya et al., 2002; Kocsy et al., 2005). Several enzymatic and non-enzymatic antioxidant defence systems control ROS concentrations to protect cells from damage (Noctor and Foyer, 1998). Sairam et al. (2000) reported that plants protect cell and subcellular systems from the cytotoxic effects of these reactive oxygen species using antioxidant enzymes such as SOD, APX, GR, CAT and metabolites like glutathione, ascorbic acid, tocopherol and carotenoids. The activity of enzymes associated with the antioxidant defence system, especially ascorbate peroxidase, has been shown to increase rapidly under heat stress in mustard (Dat et al., 1998). The membrane stability index (MSI) is a measure of membrane integrity, which is estimated in terms of the conductivity of electrolyte leakage (Sairam et al., 1997). Sairam et al. (1998; 2000) demonstrated that drought- and heat stress-tolerant genotypes show comparatively higher MSI than susceptible genotypes.

The present work was conducted to study the physiological basis of heat stress tolerance in two wheat genotypes, C 306 (tolerant to heat and water stress) and PBW 343 (susceptible to heat stress) in relation to oxidative stress and the antioxidant defence mechanism.

Materials and methods

Plant material and growth conditions

Two wheat genotypes, namely C 306, a check variety for heat and water stress tolerance screening (Sairam, 1994; Sairam et al., 1998), and PBW 343, a heat-susceptible wheat genotype released for cultivation under irrigated, timely-sown conditions in the North-Western Plain zone of India but which suffers severely due to post-anthesis high temperatures when sown late (Almeselmani, 2006), were grown in controlled environment growth chambers at the National Phytotron Facility, Indian Agricultural Research Institute, New Delhi. The seeds were sown in pots (15 cm diameter and 30 cm height) filled with a medium consisting of coco-coir peat : vermiculite : sand in a 2:1:1 ratio. The pots were irrigated with deionized water till germination, with ¼ Hoagland solution (Hoagland and Arnon, 1950) from germination to the 2–3-leaf stage and then with full strength Hoagland solution. In the control treatment, growth chamber temperatures were maintained at 23/18°C day/night throughout the experiment, while for the high temperature (HT) stress treatment temperatures were maintained at 23/18°C day/night till the maximum tillering stage (45 days after germination), and thereafter the temperatures were raised to 35/25°C day/night. On the first day of high temperature treatment (HT), the growth chamber temperature was raised gradually at a rate of approximately 1°C h^{-1} till it attained the required temperature, and was maintained at that temperature for the rest of the treatment duration. Later the plants were directly exposed to the 35/25°C day/night cycle each day. In both treatments a photoperiod of 14 h light (250 µmol $m^{-2} s^{-1}$) and 10 h dark was maintained. Samples for each estimation were collected from the two genotypes under control and heat stress conditions at the vegetative stage (one week after heat stress treatment), the anthesis stage and 15 days after anthesis.

The experiment was laid out in a completely randomized block design with four replications. The data were analysed by analysis of variance, and least significant differences (LSD) were calculated. The bar in the diagrams represents ± standard deviation. Each data point represents the mean of four replicates analysed twice, and each value is the mean of eight estimations (n=8).

Water potential estimation

Leaf water potential was measured on leaf samples in a pressure chamber (S-pms Instruments, New Delhi, India) following the method of Scholander et al. (1964).

Membrane stability index

The membrane stability index was determined by recording the electrical conductivity of leaf leachates in double distilled water at 40 and 100°C (Sairam et al., 1997). Leaf samples (0.1 g) were cut into discs of uniform size and placed in test tubes containing 10 ml of double distilled water in two sets. One set was kept at 40°C for 30 minutes and another set at 100°C in a boiling water bath for 15 minutes and their respective electric conductivities, C_1 and C_2, were measured with a Conductivity Meter (Century Instruments, Chandigarh, India). The membrane stability index (MSI) was calculated using the equation:

$$MSI = [1-(C_1/C_2)] \times 100$$

Hydrogen peroxide estimation

Hydrogen peroxide was estimated by forming a titanium-hydroperoxide complex (Rao et al., 1997). One g root material was ground with liquid nitrogen and the fine powdered material was mixed with 10 cm^3 cooled acetone in a cold room (10°C). The mixture was filtered through Whatman No. 1 filter paper, followed by the addition of 4 cm^3 titanium reagent and 5 cm^3 ammonium solution to precipitate the titanium-hydroperoxide complex. The reaction mixture was centrifuged at 10,000 rpm for 10 min in a Sigma refrigerated centrifuge (model 3K 30, Osterode, Germany). The precipitate was dissolved in 10 cm^3 2 M H_2SO_4 and then recentrifuged. The supernatant was read at 415 nm against a blank in a UV-visible spectrophotometer (model Specord Bio-200, Analytik Jena, Germany). Hydrogen peroxide contents were calculated by comparison with a standard curve drawn with known hydrogen peroxide concentrations.

Antioxidant enzyme assay

The enzyme extract for SOD, CAT, GR and POX was prepared by grinding 0.5 g leaf sample in the presence of liquid nitrogen and 10 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 0.5 mM EDTA. For the estimation of ascorbate peroxidase, the extraction buffer was further supplemented with 1 mM ascorbic acid and the pH was adjusted to 7.5. The extract was centrifuged at 15,000 rpm at 4°C for 20 min and the supernatant was used as enzyme.

The reaction mixture for superoxide dismutase estimation consisted of 20 mM methionine, 2.25 mM nitro-blue tetrazolium chloride, 3 mM EDTA, 60 µM riboflavin, 50 mM sodium carbonate, 100 mM phosphate buffer, pH 7.8, and 100 µl enzyme extract. The tubes containing the reaction mixture were kept under two 15 W fluorescent lamps for 15 min. Reaction mixture without the enzyme was used as a control. A sample containing the complete reaction mixture kept in the dark was used as a blank. Absorbance was recorded at 560 nm (Dhindsa et al., 1981). One unit of enzyme activity was taken as the amount of enzyme that reduced the absorbance reading to 50% in comparison with tubes lacking the enzyme.

Ascorbate peroxidase (APX) activity was assayed in a reaction mixture containing 3 mM ascorbic acid, 3 mM EDTA, 3 mM H_2O_2 and 33.33 mM phosphate buffer, pH 7.0. The reaction was started by the addition of H_2O_2, and the decrease in absorbance was recorded for a period of 1 min in a UV-visible spectrophotometer at 290 nm. A complete reaction mixture without ascorbic acid was used as a blank (Nakano and Asada; 1981).

Glutathione reductase activity was estimated by recording the increase in absorbance for 2 min at 412 nm. The reaction mixture contained 30 mM 5,5-dithiobis (2-nitrobenzoic acid), 20 mM oxidized glutathione, 2 mM NADPH, 20 mM phosphate buffer, pH 7.5, and 50 µl enzyme extract. Reaction mixture without oxidized glutathione was used as a blank (Smith et al., 1988).

Catalase (CAT) was assayed by monitoring the decrease in absorbance due to hydrogen peroxide at 240 nm (Aebi, 1984). The reaction mixture consisted of 50 mM potassium phosphate buffer, 12.5 mM hydrogen peroxide, 0.05 ml enzyme, and water to make up the volume to 3.0 ml. Adding H_2O_2 started the reaction and the decrease in absorbance was recorded for 1 min. Enzyme activity was computed by calculating the amount of H_2O_2 decomposed by referring to a standard curve of known concentrations of hydrogen peroxide.

Peroxidase was assayed as per Castillo et al. (1984). The reaction mixture contained 10 mM phosphate buffer, pH 6.1, 12 mM hydrogen peroxide, 96 mM guaiacol and 50 µl enzyme extract. The blank contained the complete reaction mixture without H_2O_2. The increase in absorbance was recorded for 2 min at 470 nm.

RNA isolation and gene expression analysis

Total RNA was extracted from the frozen leaf tissue using TRIZOL reagent (Invitrogen). Leaf sample (1 g) was ground in liquid nitrogen, and the powder was added to 10 mL of TRIZOL reagent in a RNAse-free centrifuge tube and incubated at room temperature for 5 min with intermittent vortexing. After adding 2 mL of chloroform, the tubes were shaken for 15 s. After 5 min of incubation at room temperature, the tubes were centrifuged at 12,000 rpm at 4°C for 15 min. The resulting upper aqueous colourless phase was transferred to a new tube, to which 5 ml of isopropanol was added. The contents of the tubes were mixed well, incubated for 10 min at room temperature and then centrifuged at 12,000 rpm at 4°C for 30 min. The resulting pellet was washed with 75% (v/v) ethanol, and the tubes were respun at 10,000 rpm at 4°C for 5 min. After removing the ethanol, the pellet was air-dried for 10 to 15 min at room temperature. The RNA was resuspended in DEPC-treated water. To eliminate DNA from the aqueous RNA extractions, samples of isolated nucleic acid were treated with 10 units of RNase-free DNase I (Qiagen, USA). Total RNA was quantified spectrophotometrically by measuring the absorbance at 260 nm. RNA was fractionated on 1% agarose gel to check the quantity and integrity.

Probes for the gene coding thylakoid-bound *APX* were amplified from wheat var. C 306 by PCR with gene-specific primers. The following gene-specific primers were designed based on the GenBank accession number AF532973:

APX – Forward Primer: GGC ATG ATT CGG GTA CAT ATG

APX – Reverse Primer: CCT GGT CCT CTG CGT ACT TC

For Northern blot analysis equal amounts of total RNA (20 µg) from normal and HT-treated plants of each genotype were fractionated on a 1.2% (w/v) denaturing formaldehyde-agarose gel as described by Sambrook et al. (1989). After electrophoresis, the RNA was capillary-blotted to nylon membranes (Hybond-N, Amersham, Arlington Heights, IL) overnight using 10× SSC. The membranes were UV-crosslinked using a Stratalinker (Stratagene) and prehybridized in 200 mM Na_2PO_4, pH 7.2, 5% (v/v) SDS, 1 mM EDTA, 10 mg/mL bovine serum albumin and 0.1 mg/mL sheared salmon sperm DNA for 4 h at 65°C. Radiolabelled probes were prepared using the HexaLabel™ DNA labelling kit (MBI Fermentas). Blots were probed with denatured ^{32}P-labelled probes added directly to the prehybridization solution at 65°C for 16 h. The blots were washed twice for 15 min at 65°C in 40 mM Na_2PO_4, pH 7.2, 5% (v/v) SDS and 1 mM EDTA, washed again for 15 min at 65°C in 40 mM Na_2PO_4, pH 7.2, 1% (v/v) SDS and 1 mM EDTA and signals were detected by exposure to Kodak X-ray films (Sigma).

Results

Leaf water potential

Water potential (ψ_w) decreased with age in both the genotypes under the two treatments, with the greatest value being recorded at the anthesis stage and the lowest 15 days after anthesis. Water potential also decreased under heat stress compared to the control treatment in both genotypes at all three stages, and the decline in ψ_w was greater in PBW 343 than in C 306 (Table 1).

Table 1

Water potential (MPa) at anthesis and 7 and 15 days after anthesis, in a heat stress-tolerant wheat genotype (C 306) and a heat stress-susceptible wheat genotype (PBW 343) grown under control (23/18°C day/night throughout the experiment) and heat stress conditions (temperature was raised to 35/25°C day/night after the maximum tillering stage)

Treatments	Genotypes	Anthesis	Anthesis+7	Anthesis+15
Control	C306	–1.88	–2.09	–2.31
	PBW 343	–2.18	–2.21	–2.42
Temp. stress	C 306	–2.08	–2.17	–2.37
	PBW 343	–2.57	–2.50	–2.84
CD at 5%	Treatment	0.033	0.038	0.066
	Genotypes	0.041	0.057	0.084
	Treatment × genotypes	0.068	0.084	0.154

Membrane stability index

The membrane stability index (MSI) decreased under heat stress in both the genotypes at all stages of growth, and a greater reduction was observed at the vegetative stage. C 306 maintained higher MSI at all the stages under heat stress compared to PBW 343 (Fig. 1). The percentage reduction in MSI under heat stress compared to normal temperature was 13, 13 and 10% at the vegetative stage, anthesis and 15 days after anthesis in C 306 and 35, 33 and 29% in PBW 343, respectively.

Hydrogen peroxide content

A significant increase in hydrogen peroxide (H_2O_2) content was recorded in both the genotypes in the heat stress treatment at all stages of growth, and the highest content in both genotypes was recorded 15 days after anthesis. However, a greater increase in H_2O_2 content was observed in the heat stress treatment in PBW 343 than in C 306 at all the stages of growth (Fig. 2). The percentage increase in H_2O_2 content under heat stress compared to normal temperature was 10, 5 and 14% at the vegetative stage, anthesis and 15 days after anthesis in C 306 and 25, 37 and 27% in PBW 343, respectively.

Antioxidant enzyme activities

There was a significant increase in all the antioxidant enzyme activities in both genotypes under heat stress conditions at all the stages of growth, but a greater increase was recorded in C 306 compared to PBW 343. Superoxide dismutase (SOD) activity increased significantly in both the genotypes under heat stress at all the growth stages and the highest SOD activity was recorded 15 days after anthesis in C 306 (Fig. 3). C 306 showed 13, 20 and 25% increases in SOD activity at the vegetative stage, anthesis and 15 days after anthesis, respectively, while PBW 343 showed 4, 7 and 11% increases over the control.

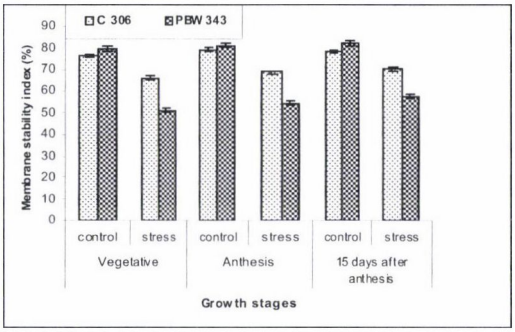

Fig. 1. Membrane stability index (%) at the vegetative stage, anthesis and 15 days after anthesis in heat stress-tolerant (C 306) and susceptible (PBW 343) wheat genotypes grown under control and heat stress conditions (control: temperature 23/18°C day/night throughout the experiment; heat stress: temperature was raised to 35/25°C day/night after the maximum tillering stage). (LSD values at $P \leq 5\%$: treatment = 6.647, genotypes = 3.713, treatment × genotypes = 12.430)

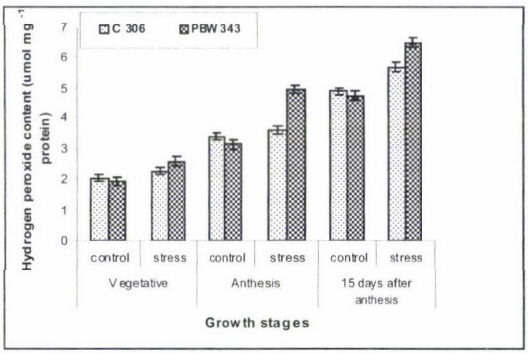

Fig. 2. Hydrogen peroxide content at the vegetative stage, anthesis and 15 days after anthesis in heat stress-tolerant (C 306) and susceptible (PBW 343) wheat genotypes grown under control and heat stress conditions (control: temperature 23/18°C day/night throughout the experiment; heat stress: temperature was raised to 35/25°C day/night after the maximum tillering stage). (LSD values at $P \leq 5\%$: treatment = 0.645, genotypes = 0.846, treatment × genotypes = 1.321)

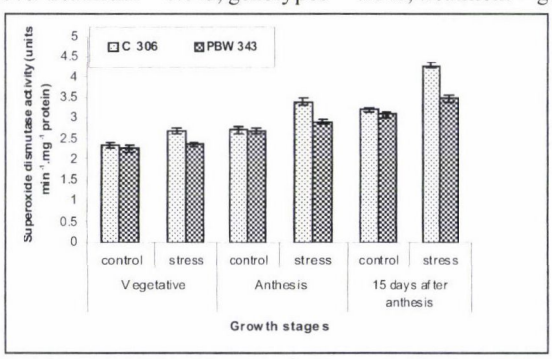

Fig. 3. Superoxide dismutase activity at the vegetative stage, anthesis and 15 days after anthesis in heat stress-tolerant (C 306) and susceptible (PBW 343) wheat genotypes grown under control and heat stress conditions (control: temperature 23/18°C day/night throughout the experiment; heat stress: temperature was raised to 35/25°C day/night after maximum tillering stage). (LSD values at $P \leq 5\%$: treatment = 0.306, genotypes = 0.571, treatment × genotypes = 0.842)

Ascorbate peroxidase (APX) activity also increased under temperature stress in both the genotypes at all stages of growth, and the values were higher in C 306 than in PBW 343 (Fig. 4). Greater APX activity was recorded 15 days after anthesis in both the genotypes. APX activity increased by 26, 35 and 30 % in C 306 at the vegetative stage, anthesis, and 15 days after anthesis, and by 14 and 16% in PBW 343 at the vegetative stage and 15 days after anthesis, respectively, while it decreased in PBW 343 at the anthesis stage by 2% under heat stress compared to control conditions.

There was a significant increase in the glutathione reductase (GR) activity in C 306 under heat stress at all stages of growth, with increases of 41, 35 and 37%, respectively, over the control. The highest GR activity in C 306 was recorded in both treatments 15 days after anthesis (Fig. 5). PBW 343 showed a reduction in the enzyme activity under heat stress compared to normal temperature at all stages of growth, and a greater reduction in enzyme activity was recorded 15 days after anthesis.

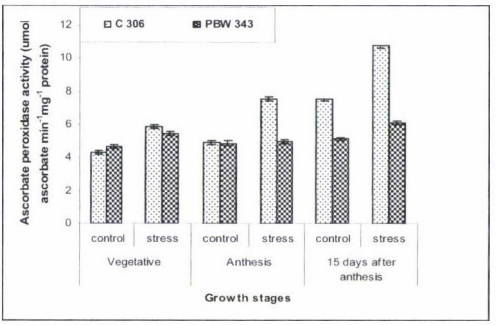

Fig. 4. Ascorbate peroxidase activity at the vegetative stage, anthesis and 15 days after anthesis in heat stress-tolerant (C 306) and susceptible (PBW 343) wheat genotypes grown under control and heat stress conditions (control: temperature 23/18°C day/night throughout the experiment; heat stress: temperature was raised to 35/25°C day/night after the maximum tillering stage). (LSD values at $P \leq 5\%$: treatment = 0.11, genotypes = 0.328, treatment × genotypes = 0.568)

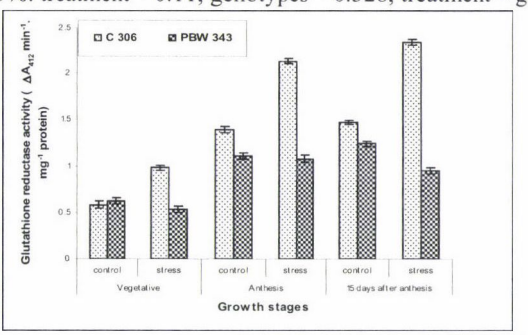

Fig. 5. Glutathione reductase activity at the vegetative stage, anthesis and 15 days after anthesis in heat-stress tolerant (C 306) and susceptible (PBW 343) wheat genotypes grown under control and heat stress conditions (control: temperature 23/18°C day/night throughout the experiment; heat stress: temperature was raised to 35/25 °C day/night after the maximum tillering stage). (LSD values at $P \leq 5\%$: treatment = 0.132, genotypes = 0.231, treatment × genotypes = 0.421)

Catalase activity also increased significantly in C 306 at all stages of growth under heat stress, the increase being 21, 24 and 24% at the vegetative stage, anthesis and 15 days after anthesis, respectively, over the control, the maximum activity being recorded 15 days after anthesis (Fig. 6). PBW 343 only showed a significant increase in catalase activity at the vegetative stage (8%), while during anthesis and 15 days after anthesis there was a 23 and 11% decline in catalase activity. In PBW 343 under both normal and heat stress conditions comparatively greater catalase activity was recorded at the vegetative and anthesis stages.

Peroxidase activity increased significantly under heat stress in C 306 at all stages of growth, and the maximum activity was observed 15 days after anthesis (Fig. 7). The percentage increase in the enzyme activity under heat stress over the control was 21, 25 and 30%, respectively. Though PBW 343 also showed the highest catalase activity 15 days after anthesis in both the treatments, heat stress only induced an increase in activity over the control at the vegetative stage (1%), while at anthesis and 15 days after anthesis there was a 17 and 24% reduction in the enzyme activity, respectively.

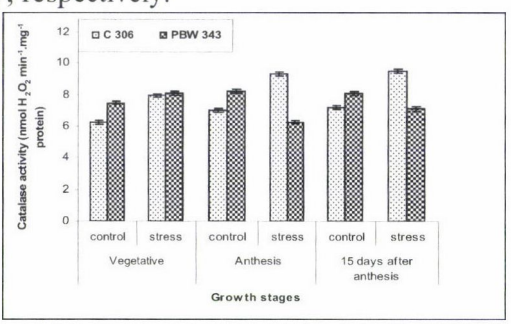

Fig. 6. Catalase activity at the vegetative stage, anthesis and 15 days after anthesis in heat stress-tolerant (C 306) and susceptible (PBW 343) wheat genotypes grown under control and heat stress conditions (control: temperature 23/18°C day/night throughout the experiment; heat stress: temperature was raised to 35/25°C day/night after the maximum tillering stage). (LSD values at $P \leq 5\%$: treatment = 0.432, genotypes = 0.893, treatment × genotypes = 1.012)

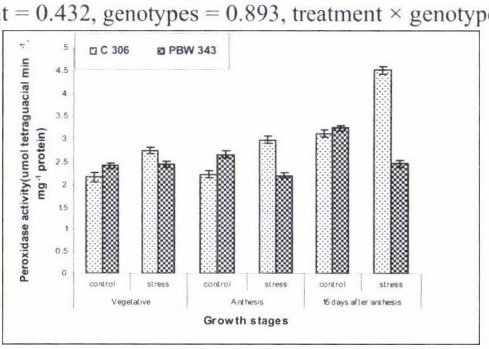

Fig. 7. Peroxidase activity at the vegetative stage, anthesis and 15 days after anthesis in heat stress-tolerant (C 306) and susceptible (PBW 343) wheat genotypes grown under control and heat stress conditions (control: temperature 23/18°C day/night throughout the experiment; heat stress: temperature was raised after maximum tillering stage to 35/25°C day/night). (LSD values at $P \leq 5\%$: treatment = 0.207, genotypes = 0.361, treatment × genotypes = 0.546)

Northern blot analysis

The *APX*-mRNA expression level increased in both the genotypes at the vegetative and anthesis stages in the heat stress treatment, but no significant differences were detected 15 days after anthesis (Fig. 8).

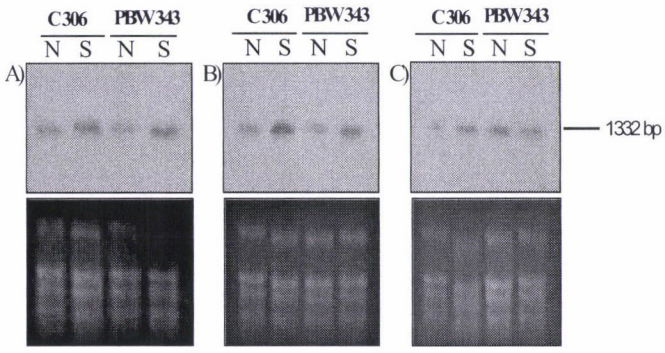

Fig 8. APX expression in response to high temperature stress in wheat genotypes differing in their heat tolerance. N: normal temperature (25/18°C day/night), S: high temperature (35/25°C day/night). Plants were exposed to high temperature stress from 45 days after sowing till maturity. Leaf samples from control and high temperature-treated plants were collected at A) vegetative stage (50 days); B) anthesis and C) anthesis+15 days. RNA was isolated and subjected to Northern blot hybridization analysis. The lower panel shows the ethidium bromide stained rRNA bands of the gel used to prepare the above RNA blot

Discussion

Crop plants experience various stresses during their life cycle, such as high or low temperature, drought and salinity, which result in the formation of various reactive oxygen species (ROS) (Sairam et al., 2000). Under unfavourable conditions excess energy that has not been used for photosynthesis may produce large amounts of ROS, which may cause oxidative damage to chloroplasts and other cell structures (Asada, 1996; Singh and Singhal, 2001). In the present study heat stress resulted in a significant increase in hydrogen peroxide content and a decline in water potential and MSI in both the genotypes at all stages. The increase in hydrogen peroxide content and the decline in water potential and MSI were greater in the susceptible genotype PBW 343. Smirnoff and Colombe (1988) suggested that the enhanced rate of hydrogen peroxide formation indicates a decrease in the capacity of the hydrogen peroxide scavenging system. Hydrogen peroxide is a toxic compound produced as a result of scavenging the superoxide radical, and its higher concentration is injurious to the cell/plant, resulting in lipid peroxidation and membrane injury (Pastori and Trippi, 1992; Baisak et al., 1994; Menconi et al., 1995). The peroxidation of membrane lipids has been observed at high temperatures (Mishra and Singhal,

1992; Upadhyaya et al., 1990), which is a symptom of cellular injury. The greater increase in H_2O_2 under high temperature stress in PBW 343 could also be the reason for higher membrane damage (low MSI). Membrane disruption may alter water, ion and organic solute movement, photosynthesis and respiration (Christiansen, 1978).

Hydrogen peroxide, though an injurious oxidant, also serves as a secondary messenger in the stress-induced, ABA-mediated signalling pathway. The role of hydrogen peroxide signalling in the induction of transcription factors associated with the induction of genes coding antioxidant enzymes has been reported by various workers (Pastori and Foyer, 2002; Agarwal et al., 2005). However, it is obvious that the greater stress-induced accumulation of H_2O_2 in PBW 343 has a more inhibitory effect, as redox signalling requires only a micromolar concentration of H_2O_2. Larkindale and Huang (2004) reported the variable influence of putative signalling components such as salicylic acid, abscisic acid, calcium, hydrogen peroxide and ethylene on antioxidant enzyme activity in *Agrostis stolonifera* var. *palustris* under heat stress.

Plants have developed enzymatic and non-enzymatic scavenging systems to quench ROS. When plants are subjected to stresses such as high temperatures, the scavenging system, in terms of antioxidant enzymes and metabolites, is not able to cope with the excess levels of ROS production, resulting in an imbalance in the production and quenching of ROS and consequently in oxidative damage (Price et al., 1989, Bowler et al., 1992; Zhang and Kirkham, 1994).

In the present study both the wheat genotypes showed increases in SOD and APX activity at all stages of growth under heat stress. The increase in SOD activity under heat stress indicates the role of SOD in the scavenging of O_2^- and the protection of the photosynthetic apparatus and demonstrates the crop/genotype's ability to tolerate stress conditions (Foster and Hess, 1982; Smirnoff, 1993). The H_2O_2 scavenging enzyme, APX, removes H_2O_2 efficiently, especially in the chloroplast, where CAT is absent (Groden and Beck, 1979). While the tolerant genotype C 306 showed a significant increase in APX activity under heat stress conditions over the control at all stages of growth, with a maximum 15 days after anthesis, PBW 343 showed only a slight increase in APX activity at the vegetative stage and 15 days after anthesis, and the level of activity was always lower than in C 306.

APX gene expression increased under heat stress conditions at the vegetative and anthesis stages in both the genotypes, though under temperature stress C 306 showed a slight increase in *APX*-gene expression 15 days after anthesis, while PBW 343 showed a decrease in gene expression in spite of the slight temperature-induced increase in APX activity level, which could be due to post-transcriptional changes in APX, resulting in a marginal increase in APX activity 15 days after anthesis.

C 306 showed an increase in the activity of GR, CAT and POX, while, PBW 343 showed a significant reduction in the activities of these three enzymes,

especially in the reproductive stages (anthesis and 15 days after anthesis). This reduction in the activity of enzymes involved in H_2O_2 scavenging could be the reason for the heat stress susceptibility of PBW 343 and could explain the significant damage experienced by the plant under high temperature conditions. GR is an important enzyme as it provides reduced glutathione and thus helps to regenerate ascorbic acid and consequently to continue the Halliwell-Asada Pathway. The decline in GR activity in PBW 343 under temperature stress ultimately affected the Halliwell-Asada Pathway, and thus adversely affected the H_2O_2 scavenging activity. The increase in GR in C 306 could protect its chloroplastic component against oxidation by H_2O_2 and minimize the potential inactivation of SOD within the chloroplasts (Foster and Hess, 1980). Catalase breaks down and detoxifies the H_2O_2 produced in mitochondria and peroxisomes. A reduction in CAT activity has been reported during short-time heat shock (Willekens et al., 1995; Foyer et al., 1997).

Comparatively lower activities of all the antioxidant enzymes in PBW 343 and the temperature-induced decline in the activities of GR, CAT and POX at anthesis and 15 days after anthesis could be attributed to the heat inactivation of these enzymes (Feierabend and Engel, 1986;Polle, 1997) as well as the failure to induce gene expression or enzyme protein synthesis (Lokhande et al., 2003) under stress conditions, which resulted in the accumulation of H_2O_2, leading to greater damage to cell membranes (Dhindsa et al., 1981), as manifested by the lower MSI in PBW 343. Chaitanya et al. (2002) also reported variations in heat stress-induced antioxidant enzyme activities between three mulberry cultivars. The greater heat inactivation of the antioxidant enzyme in the susceptible genotype PBW 343 could also be due to a deficiency in other associated mechanisms, such as osmolytes/compatible solutes, which provide protection to proteins under stress conditions (Chen and Murata, 2002).

On the other hand, the significant increase in all the antioxidant enzymes in C 306 at all stages of growth resulted in reduced oxidative damage to cell membranes under heat stress, which was further reflected in the lower hydrogen peroxide content and the less pronounced decline in MSI.

Finally, it can be concluded that the antioxidant defence mechanism plays an important role in the heat stress tolerance of wheat genotypes. The susceptibility of PBW 343 can be attributed to the lower activity of antioxidant enzymes in general and to the heat stress-induced decrease in GR, CAT and POX activities, resulting in enhanced oxidative stress, leading to damage to membranes and cellular structures, and consequently to plant growth.

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RETENTION OF PENDIMETHALIN BY HUMIC ACIDS FROM DIFFERENT FARM WASTES AND BY SOILS IN VARIOUS MANAGEMENT SYSTEMS

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The retention of pendimethalin by humic acids (HAs) from different farm wastes and from soils in various management systems was studied at 20 and 40°C. The magnitude of pendimethalin retained varied from 19.50–71.00 and 20.50–80.00 μM g^{-1} at 20°C on HAs from farm wastes and soils, respectively, which decreased with a rise in temperature. Pendimethalin retention followed the order: Soil HAs > Farm waste HAs. The highly significant values of R^2 suggested the excellent fitness of the Freundlich (0.965^{**}–0.996^{**}) and Langmuir (0.943^{**}–0.986^{**}) isotherms for pendimethalin retention. The values of the 'k' and 'n' retention parameters of the Freundlich isotherm and the 'K' and 'b' parameters of the Langmuir equation confirmed the relatively higher capacity of soil HAs to retain pendimethalin.

Key words: farm waste and soil humic acids, pendimethalin retention

Introduction

Humic acid (HA) is the alkali-soluble but acid-insoluble fraction of humic substances (HS). It interacts with herbicides, ranging from physical sorption to covalent bonding (Senesi and Testini, 1983), regulating their fate in the soil (Sha'ato et al., 2000). Such interactions are governed by the amount and characteristics of the herbicides and the HS. In this study, an attempt was made to study the retention of pendimethalin on HAs isolated from various farm wastes and from soils in different management systems.

Materials and methods

The HS were extracted from three well-decomposed organic farm wastes, namely farmyard manure, poultry litter and rice straw, and also from three soils varying in management, namely grassland, forested land and cultivated land. The samples of farm wastes and soils (after

decalcification) were repeatedly treated with 0.1N NaOH and fractionated into HA and FA (fulvic acid) by acidifying the extract. The HAs were purified by redissolving in 0.1 N NaOH, centrifuging, treating with HF+HCl, dialysing against distilled water and finally drying (Nand Ram and Raman, 1984).

Short-term batch equilibrations were conducted for the retention of pendimethalin on HAs. An aliquot from a concentrated methanol stock solution of pendimethalin (> 98%) was dissolved in a solution containing 5 mM $CaCl_2$ and 0.05mM $HgCl_2$ to prevent its biodegradation (Wolf et al., 1989), and the pH was adjusted to 5, so that HA remained in solid form (Salloum et al., 2001).

In centrifuge tubes, 20 mg HA was suspended in 50 mL of pendimethalin solution with varying concentrations (10–60 mM L^{-1}) in duplicate and equilibrated at 20°C continuously for 5 days with intermittent shaking. These tubes were then centrifuged at 2500 rpm for 10 min. The concentration of pendimethalin in the supernatant was estimated at 250 nm (λ_{max}) using a UV-VIS double beam absorption spectrophotometer. The amount of pendimethalin retained on HAs was calculated from the difference in solution concentration before and after retention, and expressed as pendimethalin retained g^{-1} HA. Similar retention studies were performed at 40°C. The blank consisted of 5 mM $CaCl_2$ and 0.05 mM $HgCl_2$. The following expressions of the Freundlich and Langmuir equations were used to evaluate the nature of pendimethalin retention on HAs of various origins:

Freundlich equation:

$$\log \frac{x}{m} = \log k + \frac{1}{n} \log C \qquad (1)$$

where x = Amount of pendimethalin retained (mM); m = Amount of humic acid (g); C = Concentration of pendimethalin in equilibrium solution (mM L^{-1}); k and n = Constants

Langmuir equation:

$$\frac{C}{x/m} = \frac{1}{Kb} + \frac{C}{b} \qquad (2)$$

where x/m = Amount of pendimethalin retained per unit amount of HA (mM g^{-1}); C = Concentration of pendimethalin in equilibrium solution (mM L^{-1}); K = Constant related to binding strength; b = Maximum amount of pendimethalin that can be retained

The retention data for pendimethalin on HAs using the Freundlich and Langmuir isotherms were statistically analysed using the JMP5 Statistical Discovery Software of the Statistical Analysis System (JMP Discovery, 2000).

Results and discussion

Retention of pendimethalin on HAs

The amount of pendimethalin retained on farm waste HAs ranged from 19.50–70.00, 19.75–70.50 and 20.00–71.00 μM g^{-1} at 20°C and from 18.37–62.50, 18.00–63.00 and 19.50–64.50 μM g^{-1} at 40°C for farmyard manure, poultry litter and rice straw, respectively. On soil HAs, it varied from 20.50–80.00, 20.25–75.00 and 20.62–75.50 μM g^{-1} at 20°C and from 20.00–75.00, 19.75–67.50 and 20.37–72.00 μM g^{-1} at 40°C for soils from grassland, forested land and cultivated land, respectively. These data inferred that adsorption increased with a successive increase in the initial pendimethalin concentration, revealing its constant partitioning between the solution phase and the adsorbent HA (Zheng and Cooper, 1996).

The magnitude of pendimethalin retention varied from 19.50–71.00 and 18.00–64.50 μM g^{-1} at 20 and 40°C, respectively, on HAs from various farm wastes. In the case of soil HAs, it ranged from 20.50–80.00 and 19.75–75.00 μM

g^{-1} at 20 and 40°C, respectively, indicating that pendimethalin retention by HAs followed the order: Soil HAs > Farm waste HAs.

Moreover, pendimethalin retention declined with a rise in temperature. This may be attributed to the weak van der Waal's forces responsible for physical adsorption. Such trends were also noticed for the adsorption of dicamba (Murray and Hall, 1989).

Fitting of pendimethalin retention on HAs

The data of pendimethalin retention on HAs of various origin were fitted to Freundlich and Langmuir equations. For the Freundlich equation, the concentration of pendimethalin in equilibrium solution (μM L^{-1}), or C, and the amount of pendimethalin retained per unit weight of HA (μM g^{-1}), or x/m, were transformed into logarithms. The Freundlich and Langmuir equations for pendimethalin retention along with their coefficients of determination (R^2) are presented in Table 1.

The values of R^2 for the Freundlich equations varied from 0.965^{**}–0.995^{**} at 20°C and from 0.985^{**} 0.996^{**} at 40°C. In the case of the Langmuir equations, the R^2 values ranged from 0.943^{**} 0.986^{**} at 20°C and from 0.960^{**}–0.982^{**} at 40°C. The highly significant values of R^2 inferred the excellent fitness of both the Freundlich and Langmuir isotherms for pendimethalin adsorption on various HAs. Other researchers also found the good fitness of either the Freundlich (Khan, 1977) or the Langmuir isotherm (Maqueda et al., 1983) or both (Gupta et al., 1985) for the retention of other herbicides on HAs. The Freundlich and Langmuir isotherms for pendimethalin retention on farmyard manure HA are depicted in Figures 1 and 2, respectively.

Table 1

Freundlich and Langmuir equations and coefficients of determination (R^2) for pendimethalin retention on HAs

Source of HA	Freundlich equation		Langmuir equation	
	log x/m	R^2	$C(x/m)^{-1}$	R^2
		20°C		
Farmyard manure	1.1758+0.4198 log C	0.965^{**}	0.1242+0.0116 C	0.945^{**}
Poultry litter	1.1382+0.4580 log C	0.995^{**}	0.1194+0.0114 C	0.951^{**}
Rice straw	1.1560+0.4504 log C	0.995^{**}	0.1154+0.0114 C	0.953^{**}
Grassland soil	1.2039+0.4756 log C	0.988^{**}	0.0889+0.0102 C	0.958^{**}
Forested soil	1.1801+0.4579 log C	0.993^{**}	0.1017+0.0109 C	0.943^{**}
Cultivated soil	1.2295+0.4450 log C	0.986^{**}	0.0784+0.0111 C	0.986^{**}
		40°C		
Farmyard manure	1.0953+0.4474 log C	0.985^{**}	0.1298+0.0133 C	0.975^{**}
Poultry litter	1.0625+0.4761 log C	0.990^{**}	0.1400+0.0125 C	0.982^{**}
Rice straw	1.1495+0.4128 log C	0.995^{**}	0.1216+0.0131 C	0.961^{**}
Grassland soil	1.1640+0.4704 log C	0.990^{**}	0.1031+0.0109 C	0.960^{**}
Forested soil	1.1584+0.4367 log C	0.996^{**}	0.1075+0.0123 C	0.977^{**}
Cultivated soil	1.1928+0.4392 log C	0.988^{**}	0.0940+0.0116 C	0.971^{**}

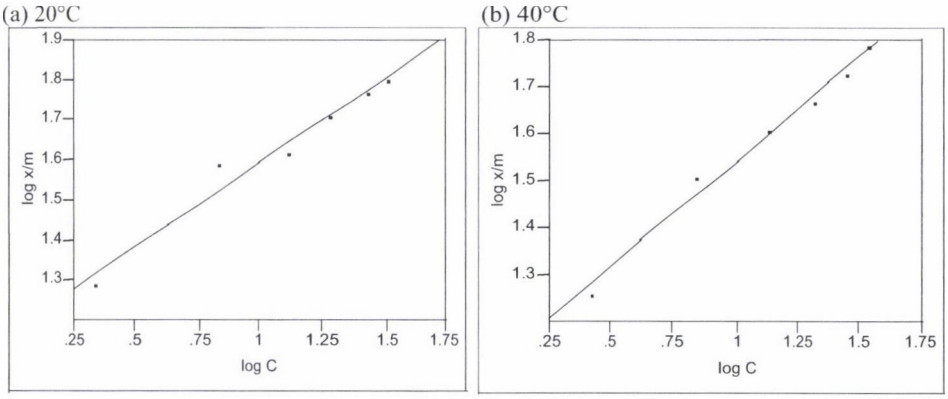

Fig. 1. Freundlich isotherms for pendimethalin retention on FYM-HA

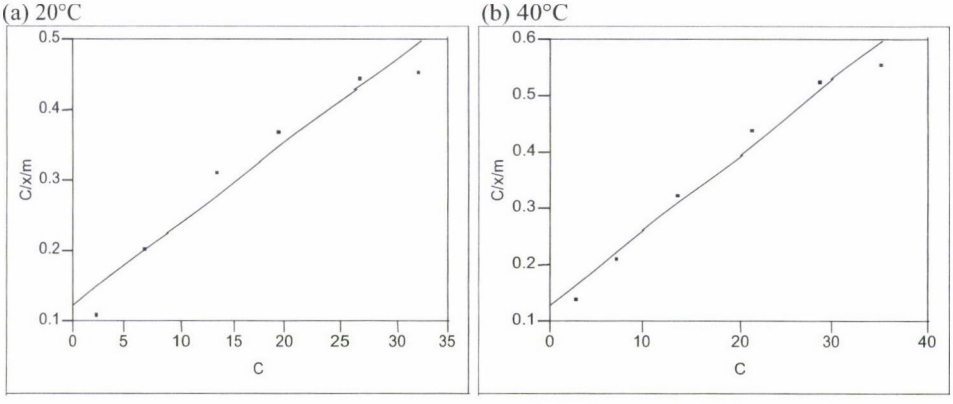

Fig. 2. Langmuir isotherms for pendimethalin retention on FYM-HA

From these results, it is evident that the retention of pendimethalin on farm waste HAs from farmyard manure, poultry litter and rice straw and on soil HAs from grassland, forested land and cultivated land was very similar in nature, possibly because of the similarity in the chemical composition of the HAs.

Retention parameters

The values of the retention parameters 'k' and 'n' for Freundlich and 'K' and 'b' for Langmuir equations are given in Table 2. The Freundlich constant 'k', a measure of retention strength, varied from 13.75–14.99 and 11.54–14.11 μM g^{-1} for HAs from farm wastes and from 13.12–16.05 and 14.40–15.59 μM g^{-1} for HAs from soils at 20°C and 40°C, respectively. The mean values of 14.35 and 12.70 μM g^{-1} for farm waste HAs, and 15.05 and 14.86 μM g^{-1} for soil HAs at the respective temperatures reflected the relatively higher capacity of soil HAs to retain pendimethalin. Moreover the 'k' values decreased with a rise in temperature. The mean 'n' values of 2.26 and 2.25 g L^{-1} for farm waste HAs and 2.17 and 2.22 g L^{-1} for soil HAs at 20°C and 40°C, respectively, showed a marginal reduction in the 'n' values of soil HAs due to their greater aromaticity (Xing, 2001).

Table 2
Retention parameters of Freundlich and Langmuir equations for pendimethalin on HAs

Source of HA	Freundlich equation				Langmuir equation			
	20°C		40°C		20°C		40°C	
	'k' ($\mu M\ g^{-1}$)	'n' ($g\ L^{-1}$)	'k' ($\mu M\ g^{-1}$)	'n' ($g\ L^{-1}$)	'K' ($L\ \mu M^{-1}$)	'b' ($\mu M\ g^{-1}$)	'K' ($L\ \mu M^{-1}$)	'b' ($\mu M\ g^{-1}$)
Farmyard manure	14.99	2.38	12.46	2.23	0.093	86.20	0.102	75.18
Poultry litter	13.75	2.18	11.54	2.10	0.095	87.71	0.089	80.00
Rice straw	14.32	2.22	14.11	2.42	0.098	87.71	0.107	76.33
Grassland soil	15.99	2.10	14.59	2.12	0.114	98.03	0.105	91.74
Forested soil	13.12	2.18	14.40	2.28	0.107	91.74	0.114	81.30
Cultivated soil	16.05	2.24	15.59	2.27	0.141	90.09	0.123	86.20

The Langmuir bonding energy constant 'K' ranged from 0.093–0.098 and 0.089–0.107 L μM^{-1} for farm waste HAs and from 0.107–0.141 and 0.105–0.123 L μM^{-1} for soil HAs at 20°C and 40°C, respectively. The mean values of 0.095 and 0.099 L μM^{-1} for farm waste HAs and 0.120 and 0.114 L μM^{-1} for soil HAs suggested the higher adsorption of pendimethalin on soil HAs. The values of 'b' (retention capacity) varied from 86.20–87.71 and 75.18–80.00 $\mu M\ g^{-1}$ for farm waste HAs and from 90.09–98.03 and 81.30–91.74 $\mu M\ g^{-1}$ for soil HAs at 20°C and 40°C, respectively. The mean 'b' values of 87.20 and 77.17 $\mu M\ g^{-1}$ for farm waste HAs and 93.28 and 86.41 $\mu M\ g^{-1}$ for soil HAs also supported the higher retention of pendimethalin by soil HAs.

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ENHANCEMENT OF ANTIOXIDANT ENZYME ACTIVITIES AND PRIMARY PHOTOCHEMICAL REACTIONS IN RESPONSE TO FOLIAR APPLICATION OF THIOLS IN WATER-STRESSED PEARL MILLET

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Primary photochemical reactions and the activities of the antioxidant enzymes chloroplastic superoxide dismutase (SOD), glutathione reductase (GR) and glutathione-S-transferase (GST) were determined in water-stressed pearl millet (*Pennisetum glaucum* L. cv. HHB-67) plants sprayed with the thiol compounds dithiothreitol (DTT), thioglycolic acid (TGA) and thiourea (TU) and the thiol modifiers 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) and N-ethylmaleimide (NEM) at the earhead emergence stage (47 days after sowing, DAS), together with a control. Sampling was done at 54 and 67 days after sowing. Photosystem I and II (PS I and II) activities (ferricyanide site) were found to increase in plants sprayed with TU, TGA and DTT at both stages (54 and 67 DAS), but a reduction in PS II activity (DCQ Site) compared with the control was caused by NEM (66.66%) and DTNB (27.77%) at 54 DAS. A similar decrease in the activity of PS II (ferricyanide site) was found at 67 DAS for DTNB (55.55%). The chloroplastic SOD activity increased in chloroplasts isolated from leaves sprayed with thiol compounds at both sampling stages, except for NEM at 54 and 67 DAS. The activities of GR and GST in the leaves were higher in thiol-treated plants than in the control at 54 and 67 DAS, while the lowest GR activity was seen for the sulphydryl modifiers (DTNB and NEM) in leaves at 54 DAS. The experimental data suggest an enhancement in the primary photochemistry and antioxidant enzyme activities of water-stressed pearl millet in response to foliar spraying with thiol compounds.

Key words: antioxidant enzymes, foliar spray, photochemistry, sulphydryl compounds, thiols

Abbreviations: $^{\cdot}O^{-}_{2}$, singlet oxygen; CAT, catalase; APOX, ascorbate peroxidase; DAS, days after sowing; DCQ, 2,6-dichloro-p-benzoquinone; DTNB, 5,5'-dithio-bis-(2-nitrobenzoic) acid; DTT, dithiothreitol; EDTA, ethylene diamine tetra acetic acid; GPOX, guaiacol peroxidase; GR, glutathione reductase; GSH, glutathione (reduced); GSSG, glutathione (oxidised); GST, glutathione-S-transferase; H_2O_2, hydrogen peroxide; NBT, nitroblue tetrazolium; NEM, N-ethylmaleimide; PCMB, p-chloro-mercuric benzoate; OH^{-}, hydroxyl radical; PS I and II, Photosystem I and II; PVP, polyvinyl pyrrolidone; ROS, reactive oxygen species; SOD, superoxide dismutase; TGA, thioglycolic acid; TU, thiourea.

Introduction

In plants exposed to drought and high temperature stress a variety of reactive oxygen species (ROS), such as superoxide radical, hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen ($^1O_2$), are formed, leading to oxidative damage (Foyer et al., 1994). The efficient detoxification of O_2^- and H_2O_2 requires the coordinated action of several antioxidant enzymes, such as catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APOX; EC 1.11.1.11), guaiacol peroxidase (GPOX; EC 1.11.1.7), superoxide dismutase (SOD; EC 1.15.1.1), glutathione reductase (GR; EC 1.6.4.2) and glutathione-S-transferase (GST; EC 2.5.1.18). The superoxide radical is converted to H_2O_2 by the action of SOD, whereas CAT converts H_2O_2 to H_2O and molecular O_2. H_2O_2 is also detoxified by peroxidases with the help of a thiol, glutathione, as reductant (Noctor and Foyer, 1998), which plays an important role as an antioxidant in cells. Tolerant genotypes of tomato and maize accumulated more GSH during chilling and had constitutively higher GR activity than sensitive ones (Walker and McKersie, 1993; Kocsy et al., 1996; 1997), corroborating the involvement of GSH and GR in increased stress tolerance. Lipid peroxidation by-products such as organic peroxides are eliminated from the cell with the help of glutathione peroxidase and GR. The non-enzymatic antioxidant defence system includes reductants such as ascorbate and glutathione. Besides antioxidant protection, the intracellular and extracellular redox states of thiols play a critical role in the stabilization of protein structure and function, the regulation of enzyme activity and the control of transcription factor activity (Deneke, 2000; Sen, 2000; Ramaswamy et al., 2007). Sulphydryl compounds such as dithiothreitol and glutathione also enhance stomatal opening in epidermal strips both in light and darkness, while the sulphydryl reagent/sulphydryl modifier N-ethyl maleimide (NEM) inhibits stomatal opening, indicating the possible involvement of sulphydryl groups in stomatal movements (Madhusudana and Anderson, 1983). Dithiothreitol (DTT) stimulated enzyme activity in the leaves of *Trillium apetalon*, but it was strongly inhibited by sulphydryl group modifiers (PCMB and iodoacetate). The sulphydryl group modifiers (PCMB and NEM) reduced photosynthetic efflux and their inhibitory effect was reversed by DTT (Fieuw and Patrick, 1993). It has been reported that compounds such as mercaptoethanol, mercaptoethylamine and thiourea improve the productivity of maize (Sahu and Solanki, 1991; Sahu et al., 1993).

Apart from playing a role in productivity these thiol compounds may also be involved in the antioxidant defence response of the plant. Sulphydryl compounds can prevent the denaturation of membrane proteins and thus help to maintain the plasma-membrane integrity under drought stress. Better yields may be obtained even under adverse environmental conditions if the plant is able to generate increased reductants via a stimulative response to oxidative stress. This paper reports the enhancement of the primary photochemistry and antioxidant defence response after foliar spraying with thiols, thus improving the stress tolerance of pearl millet.

Materials and methods

Plants, growth conditions and experimental treatments

Pearl millet plants (*Pennisetum glaucum* cv. HHB-67) were grown in the Green House Laboratory at BARC, Mumbai, in earthen pots, each filled with 5 kg of soil. Before sowing, the pots were supplied with a balanced dose of NPKS fertilizers @ 90:40:60:40 kg ha^{-1} on a dry weight basis of one furrow slice (i.e. 2.24 x 10^6/kg ha^{-1} up to 15 cm depth). Half the of nitrogen and the full dose of PKS were given at the time of pot filling and the remaining nitrogen 30 days after sowing (DAS). After fertilizer application the pots were saturated with water and allowed to settle over night. The seeds were disinfected with 70% alcohol for 2 min before sowing. Forty-two days after sowing, at the pre-flowering stage, the plants were subjected to water stress for five days. Thereafter, irrigation was provided on alternate days, when the plants usually showed symptoms of incipient wilting. This intermittent mild water stress was continued till harvest. The earhead fully emerged at 54 DAS and grain formation started at 67 DAS. The sulphydryl compounds DTT (0.07 mM), TGA (1.4 mM), TU (6.6 mM), DTNB (0.13 mM), DTNB + TU (0.13 mM + 6.6 mM), NEM (0.8 mM) and NEM + TU (0.8 mM + 6.6 mM) were sprayed at earhead emergence (47 DAS). Leaves were excised from each treatment at 54 DAS (8 days after foliar spraying) and 67 DAS (20 days after spraying) for the isolation of chloroplasts and enzymes. All the chemicals used for the experiments were procured from the Sigma Chemical Co.

Chloroplast isolation, photosystem (PS) I and II activities

Chloroplasts were isolated from pearl millet leaves following the method of Izawa and Good (1968). PS I activity was measured polarographically in a Clark-type O_2 electrode (Gilson, Saint Louis, USA) at 21°C in rate-saturating red light as methyl viologen (MeV)-mediated O_2 uptake by a chloroplast suspension equivalent to 20 µg chlorophyll (Chl) cm^{-3}, as described by Izawa (1980). PS II activity was measured as oxygen evolution by chloroplasts [20 µg(Chl) cm^{-3}] using either 0.3 mM 2,6-dichloro-*p*-benzoquinone (DCQ) or 0.4 mM potassium ferricyanide [$K_3Fe(CN)_6$] as electron acceptors, as described by Nayak et al. (2003).

Enzyme extraction and assay

Leaf samples (0.5 g fresh weight) were homogenized in ice-cold 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% polyvinylpyrrolidone. The homogenate was filtered through four layers of cheesecloth and then centrifuged at 4°C for 20 min at 15,000 g. The supernatant was collected and an appropriate aliquot/dilution of the crude extract was used for antioxidant enzyme (GR and GST) assays. All parts of the enzyme extraction were performed at 0–4°C and the enzyme assays were carried out at room temperature (23 ± 1°C), unless otherwise stated.

The chloroplastic-SOD activity was estimated in isolated chloroplasts by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT), as detailed by Becana et al. (1986). The reaction mixture (3 ml) contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 14.3 mM methionine, 82.5 µM nitroblue tetrazolium and 2.2 µM riboflavin. The system was placed 30 cm below the source of irradiance (1400 µmol photons m^{-2} s^{-1}). The reaction was run for 30 min and stopped by switching the lights off. The reduction of nitroblue tetrazolium was monitored by reading the absorbance at 560 nm. One unit of SOD (U) was defined as the amount of enzyme that produced 50% inhibition of nitroblue tetrazolium reduction under the assay conditions, as described by Giannopolitis and Ries (1977).

GR activity was measured by monitoring the decrease in absorbance at 340 nm (ε = 6.2 mM^{-1} cm^{-1}) for 1 min. The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.5 mM GSSG, 0.1 mM EDTA, 3 mM $MgCl_2$ and 0.15 mM NADPH, as described by Shaedle and Bassham (1977). Activity was expressed as µmol NADPH oxidised mg^{-1} protein min^{-1}.

GST activity was measured as per Mannervik and Guthenberg (1981) by following changes in the absorbance at 340 nm for 1 min in a mixture containing 100 mM sodium phosphate buffer (pH 6.5), 1 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene. The activity of GST was expressed as µmol 2,4-dinitrophenyl glutathione formed mg^{-1} protein min^{-1} (ε = 9.8 mM^{-1} cm^{-1}).

Statistical analysis of data

The data were statistically evaluated using a factorial completely randomized design (CRD) with three replicates (Raghavarao, 1983).

Results

PS II activity

Table 1 shows that in water-stressed plants, PS II activity was increased by foliar sprays of –SH compounds by nearly 75% (TU and TGA) and 37.50% (DTT) in comparison with the respective unsprayed control at 54 DAS, when $K_3Fe(CN)_6$ was used as an electron acceptor. When DCQ was used as electron acceptor, the PS II activity was significantly increased by DTT (30.0%) and TGA (27.77%) at 54 DAS as compared to the control. However, an inhibitory effect on PS II activity was observed for NEM (66.66%) and DTNB (27.77%) compared to the unsprayed control. This inhibitory effect was significantly reversed when TU spray was applied 24 h after DTNB and NEM. Likewise, at 67 DAS, there was a 33.33% increase in PS II activity (ferricyanide site) with foliar sprays of TU, TGA and DTT, compared to the control, while a 55.55% decline in PS II activity was observed with DTNB. It can be seen that spraying with TU significantly improved the PS II activity of DTNB-treated plants at 67 DAS, but the activity was still less than in unsprayed plants (Table 1).

PS I activity

Table 2 shows the PS I activity in chloroplasts isolated from pearl millet leaves after different treatments. Foliar spraying with thiol compounds (TU, TGA and DTT) significantly increased the PS I activity as compared to unsprayed plants. The sulphydryl modifiers (DTNB and NEM) also showed a stimulatory affect on PS I activity at both sampling dates. At 54 DAS, increased PS I activity was observed after foliar spraying with TU (52.83%) and TGA (39.62%) compared with control plants. At 67 DAS, the highest increase in PS I activity was found for TU (24%) followed by TGA (21.33%) and DTT (21.33%) as compared with control plants. Though DTNB and NEM are sulphydryl blocker reagents, they did not inhibit PS I activity except for NEM at 67 DAS (Table 2).

Table 1

Effect of foliar spraying on PS II activities (μmol O_2 evolved mg^{-1} Chl h^{-1}) in chloroplasts isolated from pearl millet. The data represent the means of three independent sets of experiments

Treatments	$H_2O \rightarrow K_3Fe(CN)_6$		$H_2O \rightarrow DCQ$
	54 DAS	67 DAS	54 DAS
Control	120	135	90
DTT (0.07 mM)	165	180	117
TGA (1.4 mM)	210	180	115
TU (6.6 mM)	210	180	95
DTNB (0.13 mM)	150	60	65
DTNB + TU (0.13 + 6.6 mM)	165	115	113
NEM (0.8 mM)	150	135	30
NEM + TU (0.8 + 6.6 mM)	152	135	65
C.D. at 5%	36.86	30.42	18.96

DAS: Days after sowing

Table 2
Effect of foliar spraying on PS I (MV → H_2O) activities (μmol O_2 consumed mg^{-1} Chl h^{-1}) in chloroplasts isolated from pearl millet. The data represent the means of three independent sets of experiments

Treatments	54 DAS	67 DAS
Control	106	150
DTT (0.07 mM)	135	182
TGA (1.4 mM)	148	182
TU (6.6 mM)	162	186
DTNB (0.13 mM)	162	185
DTNB + TU (0.13 + 6.6 mM)	150	186
NEM (0.8 mM)	136	140
NEM + TU (0.8 + 6.6 mM)	136	150
C.D. at 5%	32.46	28.23

DAS: Days after sowing

Chloroplastic SOD activity

At 54 DAS the activity of chloroplastic-SOD in the leaves (Fig. 1) increased significantly to the highest level in plants sprayed with TGA (34.27%), TU (19.72%) and DTT (19.31%) compared with unsprayed control plants. However, NEM significantly inhibited the chloroplastic SOD activity (9.53%). When the plants were sprayed with NEM followed by TU (24 h later), the inhibitory effect of NEM on enzyme activity was significantly reversed by TU. At 67 DAS foliar spraying with DTT resulted in a significant increase (18.15%) in chloroplastic SOD activity in the leaves as compared to the untreated control. In contrast the chloroplastic SOD activity declined after foliar spraying with sulphydryl group modifiers, by 19.20% for DTNB and 16.23% for NEM. However, the inhibitory effect of DTNB and NEM was significantly reversed by TU (Fig. 1).

GR activity

At 54 DAS the GR activity in the leaves increased in plants sprayed with TGA (67.42%), TU (34.38%) and DTT (27.19%) in comparison with the control (Fig. 2), while low GR activities in leaves were observed with sulphydryl group modifiers (DTNB and NEM). The reduction was significant (17.08%) for DTNB, but marginal for NEM. However, when NEM was followed by TU (after 24 h) the effect on enzyme activity was reversed (16.05%) compared with NEM alone. However, foliar spraying with TU in DTNB-treated plants did not have any reversing effect. At 67 DAS the respective increase in GR activity in the leaves was 65.15%, 48.77%, 39.36%, 26.46%, 15.66% and 13.58% for TU, DTNB + TU, DTT, NEM + TU, DTNB and NEM over the unsprayed control. No inhibitory effect on the GR activity in the leaves was observed when the plants were sprayed with sulphydryl group modifiers (DTNB and NEM), and at 67 DAS higher GR activity was observed when DTNB and NEM were followed 24 h later by TU than in the control (Fig. 2).

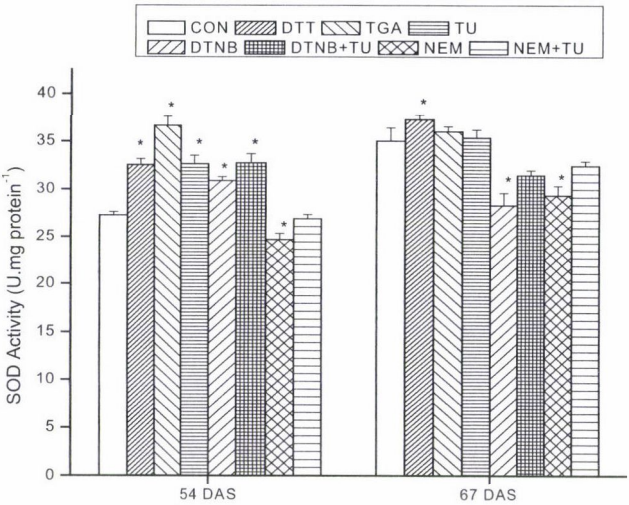

Fig. 1. Effect of foliar spraying with 0.07 mM DTT, 1.4 mM TGA, 6.6 mM TU, 0.13 mM DTNB, (0.13 + 6.6) mM DTNB + TU, 0.8 mM NEM or (0.8 + 6.6 mM) NEM + TU on the chloroplastic SOD activity in pearl millet. The data represent the means ± S.E. of three independent sets of experiments and * expresses significant changes in enzyme activities. CON: Control, DAS: Days after sowing

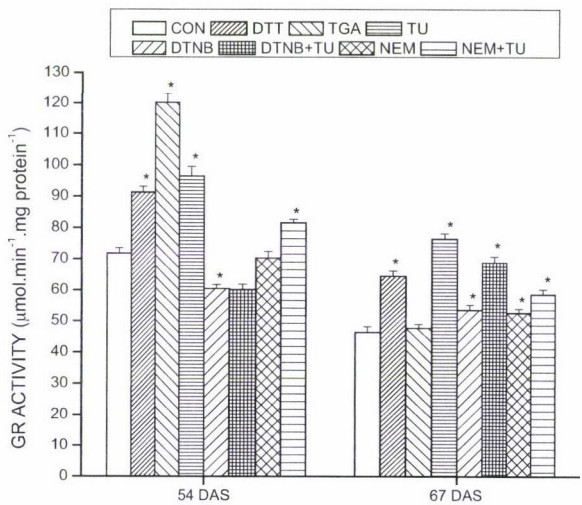

Fig. 2. Effect of foliar spraying with 0.07 mM DTT, 1.4 mM TGA, 6.6 mM TU, 0.13 mM DTNB, (0.13 + 6.6) mM DTNB + TU, 0.8 mM NEM or (0.8 + 6.6 mM) NEM + TU on the GR activity in the leaves of pearl millet. The data represent the means ± S.E. of three independent sets of experiments and * expresses significant changes in enzyme activities. CON: Control, DAS: Days after sowing

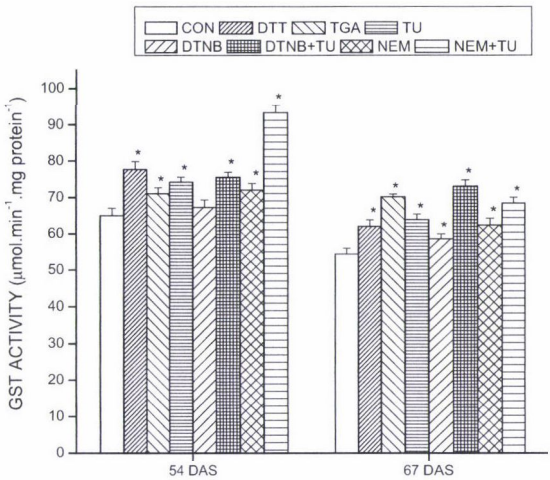

Fig. 3. Effect of foliar spraying with 0.07 mM DTT, 1.4 mM TGA, 6.6 mM TU, 0.13 mM DTNB, (0.13 + 6.6) mM DTNB + TU, 0.8 mM NEM or (0.8 + 6.6 mM) NEM + TU on the GST activity in the leaves of pearl millet. The data represent the means ± S.E. of three independent sets of experiments and * expresses significant changes in enzyme activities. CON: Control, DAS: Days after sowing

GST activity

Thiol treatments increased the GST activity in the leaves at both sampling dates (Fig. 3). At 54 DAS the increases after foliar spraying with DTT, TU and TGA were 19.46%, 14.27% and 9.41%, respectively, in comparison to the unsprayed control. The inhibitory effect of DTNB and NEM was not seen in respect of GST activity, which was higher in the NEM + TU (43.47%) and DTNB + TU (16.16%) treatments compared to the unsprayed control (Fig. 3). At 67 DAS the highest GST activity in the leaves was recorded for TGA (29.09%), followed by TU (17.45%) and DTT (14.08%), respectively, over the control. GST activity was not inhibited by sulphydryl group modifiers (DTNB and NEM) at either sampling date, so the values were higher after spraying with DTNB + TU (34.35%) or NEM + TU (25.89%) than in the unsprayed control (Fig. 3).

Discussion

It is known that drought stress induces physiological, biochemical and molecular responses in crop plants, helping them to adapt to the limiting environmental conditions (Arora et al., 2002). Photosynthesis decreases under water deficit conditions, resulting in the inhibition of electron transfer, which in turn leads to the formation of ROS that can initiate photo-oxidative damage (Asada, 1999). Thiols are thought to play a pivotal role in protecting cells

against oxidative stress. From Tables 1 and 2 it is clear that thiol treatment with TU, TGA and DTT increased the PS II and PS I activity at both sampling dates when ferricyanide/DCQ was used as the electron acceptor. However, a significant decrease in PSII activity (DCQ) was observed when sulphydryl group modifiers (DTNB, NEM) were sprayed on the plants. This inhibitory effect of DTNB and NEM was reversed by foliar spraying with TU at 54 DAS (Table 1). It should be noted that DTNB is a plasma membrane non-permeant –SH reagent, whereas NEM is a membrane permeant –SH reagent. In other words, DTNB acts on membrane-bound proteins and their –SH groups, whereas NEM can enter the cytosol and act on cytoplasmic proteins. Studies on wheat subjected to water stress indicated that the PS I and II activities were less affected by seed soaking with sulphydryl compounds (Nathawat et al., 2007). It has also been reported that foliar spraying with TU increased both canopy photosynthesis and the photosynthetically active leaf area in maize (Sahu et al., 1993). DTT has also been reported to stimulate CO_2 assimilation in the dark (Werdan et al., 1975). The redox states of thiols are involved in regulating the activity of enzymes and transcription factors, so foliar spraying with thiols may have resulted in an up-regulation of the plant antioxidant defence. In the present investigation foliar spraying with thiols (DTT, TGA and TU) significantly increased the chloroplastic SOD activity, but NEM had an inhibitory effect. In NEM-sprayed plants, TU improved the chloroplastic SOD activity at both sampling stages (Fig. 1). An increase in SOD activity was observed in pearl millet seeds pre-soaked with sulphydryl compounds during water stress (Ramaswamy et al., 2007). The over-expression of SOD was found to act as a safeguard against drought (McKersie et al., 1996) and salinity (Hasegawa et al., 2000; Zhu et al., 2002).

When biological systems dehydrate, the resulting loss in enzyme activity, such as glutathione reductase and NADPH-generating pathways, leads to an increase in the oxidative environment. In the present experiment, high thiol-mediated antioxidant enzyme activities (GR and GST) were observed in the leaves after foliar spraying with sulphydryl compounds (DTT, TGA, TU) at both sampling stages (Figs. 2 and 3). However, decreased GR activities were noted with sulphydryl reagents (DTNB and NEM) over the unsprayed control. This inhibitory effect of DTNB and NEM on enzyme activities was reversed by foliar spraying with TU at 54 DAS (Fig. 2). High GR activity increases the $NADP^+$/NADPH ratio, thereby ensuring a continuous supply of $NADP^+$ to accept electrons from the photosynthetic electron transport chain and regenerating ascorbate in the process (Noctor et al., 2002). The significantly high antioxidant enzyme activities in the thiol treatments clearly demonstrate the greater efficiency of the antioxidant system in thiol-sprayed plants. Plants have evolved cellular adaptive responses, like upregulation of the antioxidant defence system and antioxidant stress protectors (Horling et al., 2003). Tolerant genotypes of tomato and maize accumulated more GSH during chilling and had constitutively higher GR activity than sensitive ones (Walker and McKersie, 1993; Kocsy et al., 1996; 1997), corroborating the involvement of GSH and GR in increased stress tolerance.

There are few reports on the effect of thiols on the antioxidant system. In similar studies, it was shown that treatment with dimethyl thiourea was able to trap H_2O_2 and also decrease the expression of APOX in maize roots (De Zacchini and De Agazio, 2001). Dimethyl thiourea has been shown to specifically scavenge hydroxyl radicals, whereas thiourea has been widely used to study the role of hydroxyl radicals in metal-mediated biological damage both *in vitro* and *in vivo*, though most of the data available are from animal studies (Zhu et al., 2002). The long-lasting effects of foliar applied thiols may result mostly from the amplification and regulation of signal transduction pathways under drought stress. This assumption is supported by the observation of Jia and Zhang (2000) that disulphide bonds or sulphydryl groups are critical to the reactivity of signal element(s) in the signalling process under water stress and that the sulphydryl group on the cellular domain is involved in the activation of a speculated water stress receptor protein located on the plasmalemma.

The results presented here show the enhancement of the antioxidant defence system and an increase in primary photochemical reactions under drought stress in pearl millet following foliar spraying with bioactive thiols. It is still not clear how these thiols regulate the antioxidant defence mechanism at the cellular level (signal transduction pathways that mediate plant responses to water stress are poorly understood), though the activities of the antioxidant enzymes are clearly elevated. The entrapment of ROS by thiols may play an important role in this process. A better understanding of the biochemical aspects of the water stress tolerance mechanisms imparted by foliar spraying with thiols could help to increase the productivity of pearl millet in a water-limited environment.

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MILD TEMPERATURE STRESS MODULATES CYTOKININ CONTENT AND CYTOKININ OXIDASE/DEHYDROGENASE ACTIVITY IN YOUNG PEA PLANTS

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Cytokinin oxidase/dehydrogenase (CKX: EC 1.5.99.12) is able to provide a means for the rapid turnover of its substrate and it has been considered responsible for changes in the cytokinin pool in an adverse environment. Mild temperature stresses (10°C and 33°C average) were applied to young pea plants of two varieties (cvs. Manuela and Scinado) in order to assess the response of the cytokinin pool and CKX activity to altered growth conditions. Both temperature treatments increased the isopentenyl adenine (iP) and isopentenyl adenine riboside (iPR) contents in stressed plants. This trend was far more pronounced in the leaves. Low temperature additionally resulted in elevated *cis* zeatin riboside (*cis*ZR) and CKX activity. Heat did not influence the enzymatic activity in the leaves, while opposing trends were observed in the root-derived CKX activity of the two tested varieties. The data suggest that variance in the temperature provokes adaptive reactions in the cytokinin pool, which is maintained by CKX activity.

Key words: cytokinins, cytokinin oxidase/dehydrogenase, pea, temperature stress

Abbreviations: *cis*Z – *cis* zeatin, *cis*ZR – *cis* zeatin riboside, *cis*ZRP – *cis* zeatin riboside monophosphate, CK – cytokinins, CKX – cytokinin oxidase/dehydrogenase, iP – isopentenyl adenine, iPR – isopentenyl adenine riboside, iPRP – isopentenyl adenine riboside monophosphate.

Introduction

Hormonal changes are controlled by numerous signals and enzymatic pathways which govern plant acclimation to environmental stress (Mok and Mok, 2001). CK titres are usually modified by unfavourable growth conditions such as drought, water deprivation, excess salinity, changes in nutrient solutions, pathogen infection and wounding (Hare et al., 1997), high metal concentration (Atanasova et al., 2004) and herbicide treatment (Atanasova et al., 2005). Cytokinin changes, together with alterations in other endogenous plant growth regulators, may initiate physiological mechanisms involved in various protective plant stress responses.

Cytokinins are known to play an important role in several aspects of plant growth, metabolism and development at normal growth conditions. When plants are submitted to an unfavourable environment they express senescence-like symptoms (Buchanan-Wollaston et al., 2003) due to increased levels of reactive oxygen species, which prematurely provoke the process of aging (Merzlyak and Hendry, 1994). It is well known that CKs delay senescence, and their antisenescence properties are probably related to their antioxidant activity (Pauls and Thompson, 1982). Mechanisms by which environmental changes affect CKs are still not clear, but their adaptive function is indubitable.

Cytokinin oxidase/dehydrogenase (CKX), the only known enzyme that performs the degradation of adenine-type CKs, plays a significant role in the regulation of endogenous CKs. CKX catalyses the cleavage of the N6-(isopent-2-enyl) side-chain, resulting in the formation of adenine-type compounds and the corresponding isopentenyl aldehydes (Galuszka et al., 2001). Under normal growth conditions CKX maintains the homeostasis of endogenous CK levels required for plant growth and development (Kaminek et al., 1997). Relatively little data are available concerning the response of CKX activity to unfavourable environmental factors (Li et al., 2000; Manju et al., 2001; Brugière et al., 2003; Vaseva-Gemisheva et al., 2005).

This preliminary information motivated the assessment of the CK pool and the CKX activity response in young pea plants of two varieties with different vegetation periods (cvs. Manuela and Scinado), which were grown under mild suboptimal or supraoptimal temperatures (14/7°C and 38/33°C).

Materials and methods

Plant material and treatments

Fifteen-day-old plants of *Pisum sativum* L. (cv. Manuela and cv. Scinado), grown as hydroponic cultures in half-strength Hoagland's solution in a growth chamber (12/12-h photoperiod; 24/20°C day/night; photon flux density 70 μmol m^{-2} s^{-1}), were submitted to mild temperature stress for four consecutive days. The temperatures applied were 14/7 °C day/night (cold-stressed) and 38/33°C day/night (heat-stressed). Cv. Scinado, the taller variety, normally requires a shorter growing period and develops faster than cv. Manuela under the above-described control conditions (Manuela lags behind by almost one leaf stage).

Cytokinin oxidase/dehydrogenase activity assay

Enzymatic activity was measured in bulk samples (from at least 10 different plants) derived from the last fully developed leaves, i.e. the 4th leaf of cv. Manuela and the 5th leaf of cv. Scinado plants (about 0.5 g), and from secondary roots (about 2.0 g). The specific CKX activity was determined on the basis of 3-methyl-2-butenal production according to the protocol of Liberos-Minotta and Tipton (1995). The material was ground in 2.0 ml extraction buffer (pH 6.9) containing 50 mM potassium acetate, 2 mM $CaCl_2$, 1 mM $MgSO_4$ and 0.5 mM dithiothreitol. The extracts were centrifuged twice: at 15,000 rpm for 50 min, and at 15,000 rpm for 40 min after additional treatment with 0.5 mM PMSF (phenylmethylsulphonyl fluoride), 25 mg/ml streptomycin sulphate and a 0.1% solution of bovine serum albumin (BSA). The reactions for CKX activity were carried out at 37°C for 50 min in a final volume of 1.05 ml 100 mM imidazole buffer (pH 6.5) containing $CuCl_2$ and 0.050 mM isopentenyl adenine (iP). The absorbance of the samples (λ=352 nm) was measured on a Shimadzu spectrophotometer UV-1601 (Shimadzu Corporation).

Soluble protein was determined according to Bradford (1976), using bovine serum albumin as a protein standard.

The chemicals used were purchased from Sigma-Aldrich (Shaftesbury, UK).

All measurements were made in triplicate and standard error was calculated with SigmaPlot for Windows Version 8.00 Software.

Cytokinin extraction, purification and determination

Plant material derived from the last fully expanded leaves (about 1 g) and secondary roots (1.5 g) was frozen in liquid nitrogen and freeze-dried. The extraction and purification of the lyophilized samples followed the procedure described in Lexa et al. (2003). The material was extracted overnight at –20°C with Bieleski solvent (Bieleski, 1964). After adding deuterium-labelled cytokinins as internal standards (50 pmol of [$^2H_5$]Z, [$^2H_5$]ZR, [$^2H_5$]Z7G, [$^2H_5$]Z9G, [$^2H_5$]ZOG, [$^2H_5$]ZROG, [$^2H_6$]iP, [$^2H_6$]iPA, [$^2H_6$]iP7G, [$^2H_6$]iP9G, [$^2H_5$]DHZ, [$^2H_5$]DHZR; Apex Organics, Honington, UK) the extracts were centrifuged at 8,000 rpm and passed consecutively through a set of two Sep-Pak C18 cartridges (Waters Corporation, Milford, MA, USA) connected in series and, after evaporation to the water phase and adjustment of the pH to 6.5, through a DEAE Sephadex column and Sep-Pak C18 cartridges. Cytokinin bases, ribosides and glucosides were eluted twice with 80% methanol and evaporated to dryness. Cytokinin phosphates were eluted with 1 M NH_4HCO_3. These fractions were passed through Sep-Pak C18 cartridges and eluted with 80% methanol. This fraction was then evaporated to the water phase and the sample was allowed to react with alkaline phosphatase (approximately 0.5×10^{-3} units per sample) for 30 min at 37°C. After neutralisation and passage through Sep-Pak C18 cartridges, the cytokinin ribosides released from the nucleotides were eluted with 80% methanol and evaporated to dryness. The dried extracts were dissolved in 100 µl of 10% acetonitrile and filtered through centrifugal 0.2 µm Micro Spin filters (Alltech, USA). The CK fractions were then separated and quantified by HPLC (FLUX Rheos 2000 quaternary pump and CTC Analytics HTS PAL autosampler with CSI 6200 Series HPLC Oven) linked to a mass spectrometer (Finnigan LCQ) equipped with an ESI source. Samples (5 µl) were injected onto a C_{18} column (Phenomenex, AQUA, 2 mm×250 mm, 5 µm) and eluted with 0.001% acetic acid (A) and acetonitrile (B). The following gradient profile was used: 5 min at 10% B, increasing to 17% in 10 min, then to 46% in 10 min at a flow rate of 0.2 ml min^{-1}. The column temperature was kept at 30°C. The cytokinins were quantified using a multilevel calibration graph using [^{2}H]-labelled cytokinins as internal standards.

The values represent the means of liquid chromatography and mass spectrometry (LC/MS/MS) measurements in two replications.

The data presented are from two independent experiments. At least three measurements of parallel samples were performed during each experiment.

Results

Both temperature stresses resulted in increased iP and iPR content in cv. Manuela leaves (Fig. 1), but the effect was far more pronounced after the pea plants were subjected to cold, when the measured value was approximately 100 times higher than in the control. Low temperature additionally increased the *cis*ZR content. The higher substrate amount caused in the leaves by cold treatment led to raised CKX activity as well, while high temperature did not influence the enzymatic activity.

Similarly, the temperature treatments resulted in increased iP, iPR and *cis*ZR contents in cv. Scinado leaves (Fig. 2). CKX did not change after heat treatment, while low temperature stimulated the enzymatic activity almost 20-fold compared to the control.

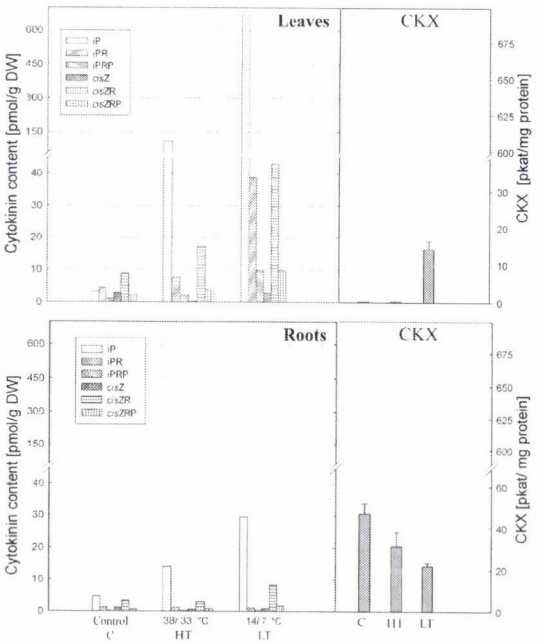

Fig. 1. Changes in specific CKX activity and endogenous cytokinin content in the last fully expanded leaves and secondary roots of cv. Manuela pea plants submitted to mild temperature stress for four days

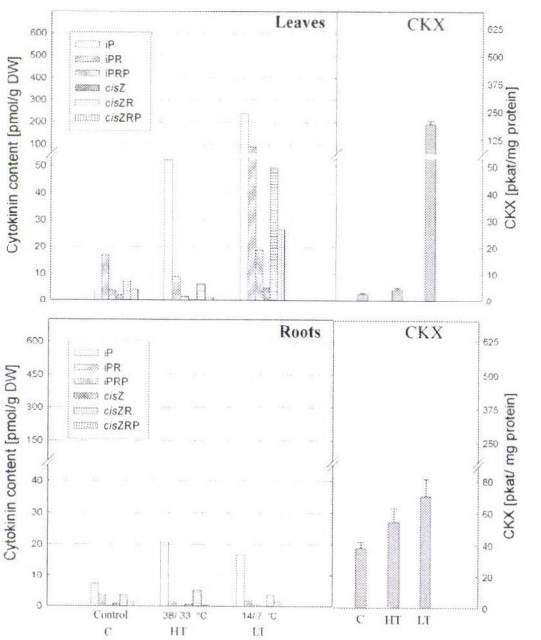

Fig. 2. Changes in specific CKX activity and endogenous cytokinin content in the last fully expanded leaves and secondary roots of cv. Scinado pea plants submitted to mild temperature stress for four days

The cytokinin content was less influenced by high temperature stress in the roots (Fig. 1 and Fig. 2). An increase was mainly observed in the iP/iPR content. Opposing trends were recorded for the CKX activity in the roots of the two cultivars. Cv. Manuela roots (Fig. 1) responded to the temperature treatments with decreased enzymatic activity. This effect was more pronounced in plants subjected to cold. Temperature stress provoked a stimulation of the enzymatic activity in cv. Scinado roots, more significant alterations in the activity again being registered at low temperature (Fig. 2).

Discussion

Temperature stresses are some of the major factors limiting plant growth and represent a significant problem in agricultural practice, as the temperature is increasing due to global warming. The mechanisms by which plants adapt to heat stress have received much attention and it has been demonstrated that, among other mechanisms, the cytokinin metabolism is involved in protecting cool-season plants from heat stress injuries (Huang, 2004).

Various plant species and varieties possess diverse potential to survive and recover after experiencing physiological stress. Plant resistance or sensitivity to unfavourable environments varies over a wide range even between the cultivars of a single species. Adverse temperatures usually provoke root inhibition, reducing the supply of water, nutrients and root-produced hormones such as CKs to the shoots, which represents one of the major factors leading to the decline in crops (Warrington and Kanemasu, 1983; Cheikh and Jones, 1994). It has been shown that the CK metabolism in the roots is most sensitive to high soil temperature (Liu et al., 2002), followed by changes in the nutrient (Tian et al., 2005) and water content (Goicoechea et al., 1995; 1996).

The mechanism by which high or low temperature affects CK levels and CKX activity is still not clear. Brugière et al. (2003) suggested that CK breakdown via CKX could be the predominant mechanism for CK inactivation under adverse environmental conditions.

The present research proved that plants tend to maintain significantly higher leaf cytokinin levels (more than 100 times the CK titres measured in unstressed tissues) at abnormal temperatures. As demonstrated earlier, approximately 100-fold higher hormonal content was registered in *IPT*-transgenic *Arabidopsis thaliana* (L.) Heynh plants, where the cytokinin biosynthetic gene was controlled by a heat-regulated constant promoter, after cyclic thermo shock (Werner et al., 2003). The subsequent metabolic inactivation of cytokinins was found to basically involve N-glucosylation. This could explain the lack of a high temperature effect on CKX activity in heat-stressed leaves in the course of the present study, where a high quantity of enzyme substrate was present. Additionally, the results obtained confirmed the suggestion of Cheikh and Jones (1994) that the maintenance of elevated

cytokinin levels under high temperature is of primary importance for plant thermotolerance. The same authors also measured higher endogenous CK content than the hormonal quantities previously identified (Lur and Setter, 1993; Morris et al., 1993).

The data obtained for cold-treated pea plants coincide with the results of Veselova et al. (2005), who detected elevated CKX activity in the aboveground parts of the plant shortly after the root zone temperature of experimental plants grown at a day/night air temperature of 26/20°C, with a nutrient medium temperature of 22/18°C was lowered to about 6°C by adding ice to the nutrient medium. Subsequently a sharp decline in the concentration of cytokinins in the shoots was registered shortly after the start of root cooling (Veselova et al., 2005). The high hormonal levels detected in temperature- stressed leaves in the present study are most probably due to the stress procedure used, which allowed adaptation to the cold over a four-day period. The high CKX activity and high iP, iPR and *cis*ZR contents in the leaves of cold-treated cv. Scinado (Fig. 2) could be discussed in terms of cultivar variations in regard to their tolerance towards suboptimal temperatures and the involvement of the cytokinin metabolism in developmental processes. The histochemical localization of cytokinin dehydrogenase by activity staining and immunochemistry (Galuszka et al., 2005) has shown that cytokinin oxidase/dehydrogenase is most abundant in the phloem cells of the shoots. CKX was confirmed to be present in the apoplast of cells, suggestive of an enzymatic capacity to control cytokinin flux through the vasculature (Galuszka et al., 2005). Cv. Scinado plants seem to respond to suboptimal temperatures with the intensified translocation of cytokinins from roots to shoots through the xylem, as evidenced by the increased CKX activity in both organs. This response to cold treatment was not observed in cv. Manuela roots, and subsequently the increase in CKX activity in the aboveground parts of the plants was not so prominent (Fig. 1).

The magnitude of cytokinin increase reported here depended on the type of plant organ and the kind of temperature stress (cold or high temperature). The cytokinin pool responded to temperature variations basically with elevated iP titres, especially in the leaves. A higher level of endogenous cytokinin may contribute to better temperature tolerance, related to the maintenance of chlorophyll content in the leaves. *Cis* zeatin was less affected by the treatments, and changes were only documented in high temperature-treated leaves, where *cis*Z was reduced compared to the controls.

The present research confirmed that cytokinin changes may influence the plant adaptation response to low and high temperature via changes in the cytokinin metabolism or in transport between roots and stems. The changes observed in the cytokinin metabolism were related to CKX activity, and this enzyme most probably plays an important role in the process of temperature adaptation.

Approaches that can increase endogenous cytokinin levels, either by the application of agents with a cytokinin-like action or by introducing favourable genes associated with the maintenance of cytokinin production, could improve the temperature tolerance of various economically important crops.

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CHANGES IN THE WATER CONTENT OF MAIZE VARIETIES AFTER PHYSIOLOGICAL MATURITY

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Changes in the water content of 22 maize varieties were investigated during the period between physiological maturity and harvesting. It was found that neither the grain moisture, the cob moisture, the moisture content of the internode below the ear nor the thousand-kernel mass changed to a statistically significant extent. No significant water uptake or drying in excess of random variation, sufficient to influence the choice of harvesting date, could be detected during the test period.

Nevertheless, considerable differences were recorded between the varieties for the moisture contents in the cob and in the internode below the ear. These could be of economic importance in choosing varieties and harvesting dates. The differences between the varieties can be attributed to the diverse genetic backgrounds, suggesting that breeding could lead to the development of maize varieties with low grain moisture at harvest.

Key words: maize, moisture content, drying

Introduction

The price of the fossil fuels required to dry maize kernels is a major item in the production costs of maize. In addition to improvements in the drying technology, breeding varieties that can be harvested at lower grain moisture content could make an important contribution to reducing the costs. The varieties widely grown 30–40 years ago flowered later and reached physiological maturity well before the grain moisture content dropped to 33%, and even when left in the field and harvested late they rarely dried to below 27–28% (Brooking 1990; Daynard et al., 1971; Hadi, 2004; Hadi and Szundy, 1990; Berzsenyi and Lap, 2006). The varieties popular today reach physiological maturity at a grain moisture content of 22–23%. The moisture content may continue to drop to 20%, or even to 15% in some varieties, by the harvest date. The dynamics of ripening prior to physiological maturity has been investigated by many authors (e.g. Hadi and Szundy, 1985; Marton, 2007), but little is known about the drying of kernels and plants after physiological maturity.

The present work was therefore carried out to determine whether the weather during the harvesting period (favourable or unfavourable for drying) or the water content of the cob, ear stalk, plant stalk or foliage had any influence on the drying of the kernels after they had reached equilibrium grain moisture content, and whether the drying costs could be reduced by the choice of harvesting date.

Materials and methods

The moisture content of the kernels, the cob and the internode below the ear was recorded in 22 hybrids, grown on six-row demonstration plots at a density of 60,000 plants/ha in Martonvásár, on a total of six occasions between November 2nd and 17th 2006. At each sampling date five plants were randomly chosen from each of five rows per variety, and the data were averaged. The grain was dried to constant weight in a drying oven at 105°C. The dry thousand-kernel mass was determined by measurements on 100 kernels × 5 plants × 2 replications. It is important to note that at the beginning of the experiment the leaves and stalk of all the plants of each variety were still green. The frost experienced on November 3rd and 4th (Table 1), combined with fusarium diseases, caused varying degrees of damage (leaf withering) to the different genotypes. Measurements on the water content of the internode below the ear were thus begun after the first symptoms became visible on November 8th. Measurements on the internode water content were not originally planned, as previous studies had revealed that the green leaves and stalk had a high water content (70–90%), which only fluctuated within the error limits, and that there were no significant differences between the varieties (Hadi and Szundy, 1990). The temperature during the test period was favourable for kernel drying, but measurable quantities of rainfall were recorded on 8 days, resulting in high humidity and providing the right conditions for the kernels to re-absorb water, if they were prone to do so.

Table 1
Rainfall and temperature data prior to harvest (Martonvásár, 20069

Date	Rainfall (mm)	t_{min} (°C)	t_{max} (°C)	Date	Rainfall (mm)	t_{min} (°C)	t_{max} (°C)
November 2	1.2	1.5	3.2	November 10	0.5	3.3	10.0
November 3	0.0	–3.0	7.0	November 11	0.0	0.0	9.8
November 4	0.2	–4.8	8.0	November 12	3.6	0.8	11.3
November 5	3.2	3.5	10.2	November 13	1.2	4.0	11.0
November 6	3.4	4.8	11.0	November 14	0.2	3.5	11.4
November 7	0.0	6.0	13.5	November 15	0.0	4.8	18.0
November 8	0.0	–0.8	14.3	November 16	0.0	5.2	16.2
November 9	0.0	5.1	13.1	November 17	0.0	5.4	18.8

Results

Averaged over the 22 varieties there was no statistically significant change in the grain moisture content during the test period. Although significant differences were recorded between the grain moisture contents of the individual varieties (Table 2), it is clear from the data that these differences already existed prior to the test period. Averaged over the 22 varieties, the equilibrium state was reached at a grain moisture content of around 18%, but this varied from 16% or

less for early varieties (Mv 251, Ipoly, Mv 277) to 20% for Majoros, Gazda and Tisza. As no significant drying was observed during the test period, indicating that the moisture content previously reached was maintained, there appeared to be a difference between the varieties in the extent of drying down after physiological maturity. This could have an influence on the harvesting date and drying costs.

There was no statistically significant change in the cob water content when averaged over the sampling dates, but differences between the varieties were significant (Table 3). The moisture content in the cobs of early varieties (Mv 251, Ipoly, Mv 277) was similar to that of the kernels, while for varieties that matured at a high grain moisture content (Majoros, Gazda, Tisza) the cob water content was high, generally above 30%.

The water content of the internode below the ear was almost 40%, averaged over the sampling dates (Table 4). This value tended to be lower for early varieties and higher for later varieties that matured at higher grain moisture content, averaged over the sampling dates. The variety Hunor was a special case, as it resembled the FAO 200 hybrids as regards the moisture content of the grain, the cob and the internode below the ear, while it behaved like the FAO 400 hybrids in terms of tasselling and silking. This suggests that Hunor contains rare, favourable genes for drying down traits that could be useful to breeders. This is confirmed by the similar behaviour of related varieties (Tarján, Koppány, Mv 500).

Table 2
Grain moisture content (%) after physiological maturity (Martonvásár, 2006)

Variety	FAO	Sampling date							
		2 Nov	7 Nov	8 Nov	10 Nov	14 Nov	17 Nov	*Mean*	CV
Mv 251	260	16.85	14.21	16.30	15.13	16.24	15.86	*15.76*	6.0
Ipoly	290	15.79	17.01	16.40	15.38	15.74	15.72	*16.01*	3.7
Mv 277	310	16.52	16.40	15.88	16.32	15.61	16.82	*16.26*	2.7
Mara	310	18.90	16.15	16.41	15.80	16.52	16.33	*16.69*	6.7
Amanita	320	18.38	18.11	16.85	16.38	15.40	16.41	*16.92*	6.7
Somacorn	340	18.97	17.63	18.76	16.62	16.82	15.21	*17.33*	8.2
Hunor	350	15.97	16.30	15.82	16.44	16.91	16.71	*16.36*	2.6
Tarján	370	16.65	16.76	15.89	18.54	16.25	16.08	*16.70*	5.8
Mv 343	380	18.40	16.42	15.16	16.30	17.00	16.14	*16.57*	6.5
MvNK 333	380	16.79	21.74	18.06	17.10	17.19	19.18	*18.34*	10.2
Mv 355	390	19.81	21.87	18.96	20.05	18.60	19.93	*19.87*	5.7
Norma	390	22.00	19.57	19.44	20.04	17.20	18.40	*19.44*	8.3
Koppány	420	20.12	17.38	16.90	17.71	18.01	19.50	*18.27*	6.9
Bogát	430	19.43	19.71	17.31	17.25	18.09	17.91	*18.28*	5.8
Mv 444	450	16.84	17.52	16.57	17.05	17.49	18.14	*17.27*	3.3
Majoros	450	22.75	19.43	24.68	22.22	20.66	21.17	*21.82*	8.4
Mv 404	450	18.92	18.75	18.66	18.01	16.91	18.45	*18.28*	4.1
Mv 437	480	18.35	19.21	20.74	18.42	18.12	18.57	*18.90*	5.1
Maraton	480	20.11	16.78	20.12	17.88	19.14	19.91	*18.99*	7.3
Gazda	480	19.55	21.86	22.11	22.93	19.61	21.07	*21.19*	6.5
Tisza	490	19.51	19.66	21.94	21.27	21.14	19.98	*20.58*	4.9
Mv 500	501	18.48	18.60	17.40	20.78	17.31	18.81	*18.56*	6.8
Mean		***18.60***	***18.23***	***18.20***	***18.07***	***17.54***	***18.24***	***18.15***	

$LSD_{5\%}$ variety mean: 1.31; $LSD_{5\%}$ sampling date: 0.68

Table 3
Cob moisture content (%) (Martonvásár, 2006)

Variety	FAO	Sampling date							
		30 Oct	2 Nov	8 Nov	10 Nov	14 Nov	17 Nov	*Mean*	CV
Mv 251	260	14.69	19.20	17.61	14.41	15.70	16.82	*16.40*	11.2
Ipoly	290	14.67	16.08	17.44	16.18	17.53	15.91	*16.30*	6.5
Mv 277	310	17.93	17.62	16.35	18.32	18.04	18.22	*17.75*	4.1
Mara	310	30.80	28.21	21.16	15.31	20.40	18.80	*22.45*	26.2
Amanita	320	18.12	9.86	19.76	18.23	16.98	17.89	*16.81*	20.9
Somacorn	340	20.13	24.29	26.91	19.23	20.49	17.06	*21.35*	16.8
Hunor	350	19.97	19.12	16.54	18.19	19.94	16.94	*18.45*	8.0
Tarján	370	24.37	18.64	16.86	25.38	18.93	15.96	*20.03*	19.6
Mv 343	380	15.48	20.61	14.36	19.41	21.83	16.69	*18.06*	16.6
MvNK 333	380	37.41	25.16	28.83	23.55	23.46	29.73	*28.02*	18.9
Mv 355	390	39.89	36.91	31.39	30.84	29.40	28.09	*32.75*	14.4
Norma	390	23.45	36.77	30.84	35.29	23.01	27.02	*29.40*	20.0
Koppány	420	25.40	34.76	22.31	21.61	26.76	29.61	*26.74*	18.3
Bogát	430	30.85	37.51	24.73	24.46	30.48	24.06	*28.68*	18.5
Mv 444	450	19.47	19.68	20.54	19.78	21.19	20.06	*20.12*	3.2
Majoros	450	25.41	37.05	46.41	33.46	38.61	33.38	*35.72*	19.4
Mv 404	450	26.67	30.68	29.57	26.40	20.45	26.85	*26.77*	13.3
Mv 437	480	33.59	28.54	36.90	31.29	31.72	28.44	*31.74*	10.1
Maraton	480	35.06	30.36	35.95	28.98	34.07	29.34	*32.29*	9.6
Gazda	480	38.50	32.49	41.58	43.98	33.06	33.37	*37.16*	13.2
Tisza	490	47.02	31.11	37.08	32.70	37.73	22.73	*34.73*	23.3
Mv 500	501	30.50	26.87	23.65	32.30	21.14	23.93	*26.40*	16.4
Mean		***26.79***	***26.43***	***26.22***	***24.97***	***24.59***	***23.22***	***25.37***	

$LSD_{5\%}$ variety mean: 4.85; $LSD_{5\%}$ sampling date: 2.53

Table 4
Water content of the internode below the ear (Martonvásár, 2006)

Variety	FAO	Sampling date					
		8 Nov	10 Nov	14 Nov	17 Nov	*Mean*	CV
Mv 251	260	44.08	36.68	29.91	37.88	*37.14*	15.6
Ipoly	290	43.35	40.09	20.00	31.72	*33.79*	30.8
Mv 277	310	30.25	30.33	22.03	26.04	*27.16*	14.6
Mara	310	46.55	18.87	35.90	29.31	*32.66*	35.6
Amanita	320	46.03	45.33	34.78	37.66	*40.95*	13.7
Somacorn	340	38.96	46.34	41.05	28.40	*38.69*	19.5
Hunor	350	22.22	21.00	25.23	20.21	*22.17*	9.9
Tarján	370	25.69	50.00	31.48	34.44	*35.40*	29.3
Mv 343	380	18.75	36.80	42.00	25.23	*30.69*	34.6
MvNK 333	380	36.05	44.97	31.76	40.82	*38.40*	14.9
Mv 355	390	55.32	57.94	52.33	39.68	*51.32*	15.8
Norma	390	58.05	55.43	29.41	46.15	*47.26*	27.4
Koppány	420	30.72	51.12	38.13	39.58	*39.89*	21.1
Bogát	430	51.08	35.00	29.91	39.04	*38.76*	23.3
Mv 444	450	26.85	27.43	21.15	28.87	*26.08*	13.0
Majoros	450	63.14	56.14	55.07	45.74	*55.02*	13.0
Mv 404	450	48.41	31.86	27.03	28.87	*34.04*	28.7
Mv 437	480	61.99	68.91	66.67	59.72	*64.32*	6.5
Maraton	480	31.34	37.62	33.33	36.97	*34.82*	8.6
Gazda	480	39.82	63.16	43.01	51.61	*49.40*	21.1
Tisza	490	41.10	41.07	32.00	35.57	*37.44*	11.9
Mv 500	501	47.59	52.25	40.94	42.51	*45.82*	11.2
Mean		***41.24***	***43.11***	***35.60***	***36.64***	***39.15***	

$LSD_{5\%}$ variety mean: 10.13; $LSD_{5\%}$ sampling date: 4.32

In the case of thousand-kernel mass (Table 5), the significant differences between the varieties were to be expected. No significant differences were found between the sampling dates, averaged over the 22 varieties, indicating that during the experimental period there was no further increase in thousand-kernel mass, but also no significant reduction.

Table 5
Thousand-kernel mass after physiological maturity (Martonvásár, 2006)

Fajta	FAO	Sampling date						
		2 Nov	7 Nov	8 Nov	10 Nov	14 Nov	17 Nov	*Mean*
Mv 251	260	289.0	326.0	308.0	301.6	291.5	317.4	*305.6*
Ipoly	290	308.0	327.0	318.5	333.7	289.0	310.7	*314.5*
Mv 277	310	311.5	308.5	304.5	341.0	332.5	324.0	*320.3*
Mara	310	305.5	296.0	303.0	293.0	341.0	287.0	*304.3*
Amanita	320	356.5	330.0	335.5	342.0	335.0	351.5	*341.8*
Somacorn	340	328.0	343.5	327.0	303.5	311.5	329.0	*323.8*
Hunor	350	305.0	303.0	306.0	302.5	307.0	304.0	*304.6*
Tarján	370	285.0	293.0	307.0	347.0	299.0	323.5	*309.1*
Mv 343	380	376.5	338.5	319.0	346.5	312.5	330.0	*337.2*
MvNK 333	380	333.5	333.0	329.0	349.0	306.0	314.0	*327.4*
Mv 355	390	346.5	355.5	350.5	331.0	378.5	335.5	*349.6*
Norma	390	376.0	394.5	358.5	367.0	378.0	372.5	*374.4*
Koppány	420	325.5	366.0	354.0	290.5	341.5	307.5	*330.8*
Bogát	430	366.0	334.0	387.0	343.0	412.0	330.0	*362.0*
Mv 444	450	368.0	395.5	410.5	411.0	349.0	379.0	*385.5*
Majoros	450	340.5	354.5	319.0	329.0	338.0	303.5	*330.8*
Mv 404	450	284.5	312.0	298.5	300.5	287.5	289.5	*295.4*
Mv 437	480	318.0	286.0	290.5	299.0	291.5	296.0	*296.8*
Maraton	480	400.5	379.5	341.5	367.5	357.0	370.0	*369.3*
Gazda	480	377.0	429.0	357.5	363.0	393.5	391.5	*385.3*
Tisza	490	324.0	335.0	363.0	360.7	332.0	342.4	*342.9*
Mv 500	501	315.5	350.0	330.0	369.0	341.5	334.2	*340.0*
Mean		***333.7***	***340.5***	***332.6***	***336.0***	***333.0***	***329.2***	***334.1***

$LSD_{5\%}$ variety mean: 21.56; $LSD_{5\%}$ sampling date: 11.26

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EFFECT OF RHIZOBIAL INOCULATION ON GROWTH, YIELD, NODULATION AND BIOCHEMICAL CHARACTERS OF VEGETABLE PEA (*Pisum sativum*)

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A field experiment was conducted with 8 genotypes of vegetable pea and 3 levels of rhizobial inoculation for two consecutive years. Inoculation with *Rhizobium leguminosarum* @ 20 g kg^{-1} seed made the plants taller and enhanced biomass production through branching, but delayed maturity. It resulted in more pods, enlarging them to contain more seeds, leading to higher test weight and enhanced harvest index. It increased nodulation, the benefit of which was reflected in most of the yield traits. It also enriched the seeds with nitrogen and protein, as well as activating nitrogenase enzyme in the root nodules to fix more atmospheric nitrogen.

Key words: nodulation, rhizobial inoculation, vegetable pea, yield, biochemical characters

Introduction

Vegetable pea (*Pisum sativum* L. var. *hortense*) is grown for the green pods containing immature seeds on the plains of northern India during winter and on the hills in summer. It is grown on 0.2726 m ha mostly in Uttar Pradesh, Bihar, Punjab, Himachal Pradesh, Orissa and Karnataka states, with an annual production of 2.712 m t and productivity of 9.9 t ha^{-1} constituting about 4.5% of the area and 2.9% of the total vegetable production in the country (Anonymous, 2001). Uttar Pradesh alone accounts for 0.1504 m ha area (55.2%) under vegetable pea, producing 1.884 m t (69.5%) with a productivity of 12.5 t ha^{-1} However, Uttarakhand, a state newly carved out of Uttar Pradesh, has a vegetable pea area of only 0.0116 m ha and produces 0.069 m t with a productivity of only about 6.0 t ha^{-1} (Arora and Singh, 2002).

Being a legume, pea meets most of its nitrogen needs by fixing atmospheric nitrogen in root nodules through a symbiotic relationship with *Rhizobium leguminosarum* (Vance, 1997), thus lessening the demand for

fertilizers. The symbiotic efficiency is dependent on the *Rhizobium* strain, the host plant, soil conditions such as acidity and nutrient status, and the environment (Vincent, 1974). Therefore, a legume must be inoculated with a suitable rhizobial strain to ensure effective nodulation, not only to meet the nitrogen needs of the legume but also to benefit the growth of the subsequent crop and improve soil fertility. Since the formation and maintenance of nodules regulate nitrogen fixation, the degree of nodulation in legumes is used as a measure of symbiotic activity.

Seed inoculation is the most popular and convenient way of rhizobial inoculation, but the seed must be covered thoroughly with the inoculum. Native rhizobial strains compete with those applied through inoculums. Under such situations, the rhizobial strains applied to the seed at recommended rates may be insufficient to compete successfully with indigenous rhizobia. Thus, it was hypothesized that an increase in the inoculation rate might solve this problem. Considering the importance of rhizobial inoculation in legume production as well as the small area and poor productivity of pea in Uttarakhand, it was thought to be desirable to screen genotypes of vegetable pea for their atmospheric nitrogen fixation efficiency. Therefore, the present study was carried out in the Indo-gangetic plains of Uttarakhand, lying in northern India, to evaluate eight genotypes of vegetable pea grown with three levels of rhizobial inoculation applied to the seed.

Materials and methods

A field experiment was conducted for two consecutive years in the winter season of 2004–05 and 2005–06 at R. M. P. P. G. College, Gurukul Narsan, district Haridwar, which lies in the plains of the Garhwal division of Uttarakhand State in northern India and has a semi-arid climate. The experimental soil in both years had sandy loam texture, normal pH, no salinity, low contents of organic carbon, available N and K, but medium status for available P (Table 1), as determined using standard methods.

The seeds of vegetable pea were sown @ 100 kg ha^{-1} at a distance of 30 cm between rows and 10 cm between seeds on 5th and 1st November in 2004 and 2005, respectively. The treatments included eight genotypes of vegetable pea, namely Arkel, Pant Sabji Matar 3 (PSM 3), PSM 4, Punjab Matar Ageta 6 (PMA 6), VL 7, Azad P 1, Azad P 3 and NDVP 12; and three levels of rhizobial inoculation: 0, 20 and 40 g kg^{-1} seed. The treatments were replicated twice in a factorial randomized block design (RBD) having an individual plot size of 4 × 3 m. The recommended rate of inoculation is 20 g kg^{-1}, but to test the hypothesis an increased rate of 40 g kg^{-1} was also applied. At the time of sowing, N, P and K were applied as basal fertilizers @ 20, 26 and 33 kg ha^{-1}, respectively. Standard agronomic practices were followed throughout the experiments.

Table 1
Characteristics of the soils of the experimental fields in the two years of the experiment

Soil characteristics	2004–05	2005–06
Soil pH	7.40	7.00
Electrical conductivity (dS m^{-1})	0.36	0.30
Organic carbon (g kg^{-1} soil)	4.50	3.80
Available N (kg ha^{-1})	248.00	220.00
Available P (kg ha^{-1})	12.80	10.50
Available K (kg ha^{-1})	300.00	260.00

Before sowing, the seeds of each pea genotype were inoculated with a charcoal-based culture of *Rhizobium leguminosarum*. For this, 10% jaggery solution was prepared in water, followed by heating and cooling, and then sprinkled on the pea seeds. Afterwards, the amount of rhizobial culture required for each level of inoculation was broadcast on the seeds and mixed thoroughly to cover the entire surface of the seeds with culture. Observations were made for the following growth and yield traits (5 each) as well as nodulation and biochemical characters (3 each) on five randomly selected uniform plants at different growth stages:

Growth characters: Plant height/plant at maturity, biomass/plant at 50% flowering, number of branches/plant at maturity, percentage flower shedding and days to maturity.

Yield characters: Number of pods/plant, pod length/plant, number of seeds/pod, test weight and harvest index.

Nodulation characters: Number of nodules/plant at 75% flowering, nodule dry weight at 75% flowering and root dry weight/plant at 50% flowering.

Biochemical characters: Seed nitrogen, seed protein, and nitrogenase activity. The acetylene reduction assay (ARA) described by Hardy et al. (1973) was used to estimate the nitrogenase activity in intact root nodules at 75% flowering.

The data for all the characters were statistically processed using factorial RBD analysis (Panse and Sukhatme, 1969).

Results and discussion

The mean response to rhizobial inoculation for each character was computed by subtracting the control (uninoculated) value from the mean of the inoculated levels for each genotype of vegetable pea. The response at each inoculation level (20 and 40 g kg^{-1} seed) was computed by subtracting the value of the uninoculated treatment (0 g kg^{-1} seed).

Growth characters

Seed inoculation with *Rhizobium leguminosarum* benefited all growth characters except flower shedding (Table 2a, b). It made the plants taller and resulted in greater biomass and more branches, but delayed maturity. Plant height rose by 3.70–7.67% and 4.18–9.34% over the control due to inoculation during 2004–05 and 2005–06, respectively. Inoculation at 20 and 40 g kg^{-1} seed made the plants taller by 4.29 and 5.50% in the first year and 5.40 and 6.28% in the second year, respectively, and increased biomass by 8.26–22.16% and 8.01–23.29% over the control in 2004–05 and 2005–06, respectively. Biomass production was enhanced by 15.92 and 19.10% in the first year, and 14.80 and 19.02% in the second year by inoculation at 20 and 40 g kg^{-1} seed, respectively. This is attributed to the beneficial impact of rhizobial inoculation on pea plant growth (Prasad and Maurya, 1993).

Inoculation produced more branches, ranging from 13.63–17.61% in 2004–05 and from 9.23–22.42% in 2005–06 compared with the control. Doses of 20 and 40 g kg^{-1} seed of inoculation enhanced the number of branches by 11.54 and 18.42% in the first year and by 13.03 and 20.27% in the second year, respectively. Moreover, inoculation delayed the maturity of pea by 1–2 days in both years. Enhanced nitrogen availability in the soil due to inoculation with *Rhizobium* had a beneficial impact on growth characters (Patel et al., 1998).

Table 2a
Effect of rhizobial inoculation on plant height, biomass at 50% flowering and number of branches/plant of vegetable pea

Genotype	2004–05				2005–06			
	0	20	40	Mean	0	20	40	Mean
				Plant height (cm)				
Arkel	59.40	62.20	63.30	61.63	58.00	61.00	61.60	60.20
PSM 3	61.65	65.10	66.25	64.33	59.40	63.50	64.10	62.33
PSM 4	80.20	83.39	84.00	82.53	77.30	82.00	82.60	80.63
PMA 6	48.20	51.30	52.50	50.66	46.00	50.00	50.60	48.86
VL 7	57.30	59.50	60.60	59.13	56.20	58.80	59.50	58.16
AP 1	79.50	82.55	83.10	81.71	81.89	84.90	85.75	84.18
AP 3	77.30	80.10	80.60	79.33	75.20	78.50	79.00	77.56
NDVP 12	78.30	81.00	81.40	80.23	74.60	78.60	78.70	77.26
Mean	67.73	70.64	71.46		66.05	69.66	70.23	
	V	I	V × I		V	I	V × I	
SEM ±	0.53	0.32	0.91		0.37	0.23	0.65	
C.D. 5%	1.55	0.95	NS		1.10	0.67	NS	
				Biomass at 50% flowering (g/plant)				
Arkel	11.90	13.90	14.35	13.38	11.25	12.80	13.60	12.55
PSM 3	12.50	14.40	14.90	13.93	11.90	14.00	14.70	13.53
PSM 4	15.50	18.40	18.90	17.60	15.30	17.60	18.40	17.10
PMA 6	10.60	12.60	13.30	12.16	10.90	13.30	13.60	12.60
VL 7	11.00	12.70	12.90	12.20	10.80	12.30	12.70	11.93
AP 1	13.80	16.00	16.50	15.43	13.40	15.50	16.30	15.06
AP 3	12.70	13.70	13.80	13.40	13.10	14.10	14.20	13.80
NDVP 12	12.50	14.80	15.10	14.13	12.50	14.50	14.80	13.93
Mean	12.56	14.56	14.96		12.39	14.26	14.78	
	V	I	V× I		V	I	V × I	
SEM ±	0.13	0.08	0.22		0.17	0.18	0.30	
C.D. 5%	0.38	0.23	NS		0.52	0.54	NS	
				Number of branches/plant				
Arkel	9.00	9.80	10.80	9.86	9.30	9.90	10.65	9.95
PSM 3	10.90	12.30	12.75	11.98	10.50	12.00	12.50	11.66
PSM 4	11.75	13.10	14.05	12.96	13.00	14.10	14.30	13.80
PMA 6	8.80	10.20	10.50	9.83	8.90	9.70	11.10	9.90
VL 7	8.80	9.70	10.30	9.59	9.10	10.10	10.70	9.96
AP 1	11.20	12.40	13.10	12.23	10.70	12.90	13.30	12.30
AP 3	11.10	12.40	13.40	12.30	10.90	12.70	13.80	12.46
NDVP 12	11.00	12.20	12.90	12.03	10.50	12.30	13.40	12.06
Mean	10.31	11.51	12.23		10.36	11.71	12.46	
	V	I	V × I		V	I	V × I	
SEM ±	0.16	0.10	0.28		0.14	0.08	0.24	
C.D. 5%	0.48	0.29	NS		0.41	0.25	NS	

NS: Non-significant; V: Variety; I: Inoculation level

The year had no significant effect on the growth parameters. However, among the genotypes, PSM 4 performed best for plant height, biomass production and branching, while Arkel performed poorly in both years.

Table 2b
Effect of rhizobial inoculation on flower shedding and days to maturity of vegetable pea

Genotype	2004–05				2005–06			
	0	20	40	Mean	0	20	40	Mean
				Flower shedding (%)				
Arkel	1.00	0.50	0.50	0.66	1.00	0.50	0.50	0.66
PSM 3	1.00	0.50	0.50	0.66	1.00	0.50	0.50	0.66
PSM 4	1.00	0.50	0.50	0.66	1.00	0.50	0.50	0.66
PMA 6	1.00	1.00	0.75	0.91	1.00	1.00	0.50	0.83
VL 7	1.00	1.00	0.50	0.83	1.00	1.00	0.50	0.83
AP 1	1.00	0.50	0.50	0.66	1.00	0.50	0.50	0.66
AP 3	1.00	1.00	0.50	0.83	1.00	0.50	0.50	0.66
NDVP 12	0.50	1.00	1.00	0.83	1.00	1.00	0.50	0.83
Mean	0.93	0.75	0.59		1.00	0.68	0.50	
	V	I	V × I		V	I	V × I	
SEM ±	0.19	0.12	0.34		0.20	0.12	0.36	
C.D. 5%	NS	NS	NS		NS	NS	NS	
				Days to maturity				
Arkel	104.00	105.00	106.00	105.00	104.50	105.00	106.00	105.16
PSM 3	117.00	117.00	119.00	117.66	116.00	117.00	119.00	117.33
PSM 4	114.00	115.00	116.50	115.16	115.00	115.50	116.00	115.50
PMA 6	104.00	105.00	105.00	104.66	104.00	104.50	105.00	104.50
VL 7	104.00	105.50	106.00	105.16	104.00	105.50	106.00	105.16
AP 1	125.00	126.50	127.50	126.33	126.00	127.00	128.00	127.00
AP 3	118.00	119.00	120.00	119.00	119.00	119.50	120.00	119.50
NDVP 12	109.00	110.00	111.00	110.00	109.50	110.00	112.00	110.50
Mean	111.87	112.87	113.87		112.25	113.00	114.00	
	V	I	V × I		V	I	V × I	
SEM ±	0.63	0.38	1.09		0.46	0.28	0.80	
C.D. 5%	1.85	1.13	NS		1.36	0.83	NS	

NS: Non-significant; V: Variety; I: Inoculation level

Yield parameters

Rhizobial inoculation benefited the yield characters by producing more pods per plant, enlarging their length to give a greater number of seeds per pod, increasing the test weight, and ultimately improving the harvest index (Table 3a, b).

The percentage increase in harvest index due to inoculation in comparison to no inoculation ranged from 2.01 (NDVP 12) to 10.56 (PMA6) in 2004–05, and from 0.88 (AP1) to 11.29 (AP3) in 2005–06. The levels of inoculation also increased the mean harvest index by 5–7% over the control. The vigorous plant growth, with more branches and fertile flowers, and the greater number of pods after inoculation were responsible for the increase in yield parameters (Feng et al., 1997). Kanaujiya et al. (1997) also reported a significant increase in pea yields due to seed inoculation with *Rhizobium leguminosarum*. This could be attributed to the better growth of inoculated pea plants, translating into greater seed yield.

Table 3a
Effects of rhizobial inoculation on number of pods/plant, pod length and number of seeds/pod of vegetable pea

Genotype	2004–05				2005–06			
	0	20	40	Mean	0	20	40	Mean
				Number of pods/plant				
Arkel	9.10	9.90	10.10	9.70	9.40	10.70	10.90	10.33
PSM 3	7.50	8.50	8.60	8.20	7.20	8.30	8.50	8.00
PSM 4	10.60	11.70	11.90	11.40	10.40	11.50	11.90	11.26
PMA 6	6.60	7.40	7.60	7.20	6.40	7.50	7.80	7.23
VL 7	6.00	6.90	7.30	6.73	5.70	6.50	6.90	6.36
AP 1	7.20	8.50	8.60	8.10	7.00	7.80	8.20	7.66
AP 3	7.10	8.00	8.20	7.76	7.00	7.90	8.10	7.66
NDVP 12	8.90	9.70	9.90	9.50	8.10	9.50	9.80	9.13
Mean	7.87	8.82	9.02		7.65	8.71	9.01	
	V	I	V × I		V	I	V× I	
SEM ±	0.12	0.07	0.20		0.30	0.18	0.52	
C.D. 5%	0.35	0.21	NS		0.88	0.54	NS	
				Pod length (cm)				
Arkel	8.00	8.30	8.40	8.23	8.00	8.20	8.29	8.16
PSM 3	8.29	8.60	8.70	8.53	8.40	8.50	8.60	8.50
PSM 4	7.80	8.20	8.29	8.10	7.60	7.90	8.10	7.86
PMA 6	8.00	8.29	8.40	8.23	8.10	8.30	8.50	8.30
VL 7	7.50	7.70	7.90	7.70	7.50	7.80	7.90	7.73
AP 1	8.29	8.50	8.60	8.46	8.20	8.40	8.50	8.36
AP 3	7.90	8.20	8.29	8.13	8.10	8.40	8.50	8.33
NDVP 12	8.20	8.40	8.50	8.36	8.10	8.50	8.60	8.40
Mean	8.00	8.27	8.38		8.00	8.25	8.37	
	V	I	V × I		V	I	V× I	
SEM ±	0.065	0.040	0.114		0.083	0.051	0.145	
C.D. 5%	0.19	0.12	NS		0.24	0.15	NS	
				Number of seeds/pod				
Arkel	6.60	6.90	7.05	6.85	6.30	6.70	6.85	6.61
PSM 3	7.20	7.50	7.60	7.43	6.85	7.20	7.35	7.13
PSM 4	6.80	7.15	7.25	7.06	6.60	6.95	7.10	6.88
PMA 6	6.20	6.50	6.65	6.44	6.00	6.30	6.45	6.25
VL 7	5.70	6.00	6.10	5.93	5.50	5.80	5.95	5.75
AP 1	7.30	7.60	7.70	7.53	6.90	7.25	7.40	7.18
AP 3	6.50	6.80	6.95	6.75	6.40	6.80	6.90	6.70
NDVP 12	7.00	7.30	7.40	7.23	6.80	7.00	7.25	7.06
Mean	6.66	6.96	7.08		6.42	6.75	6.90	
	V	I	V × I		V	I	V × I	
SEM ±	0.089	0.054	0.154		0.077	0.050	0.134	
C.D. 5%	0.26	0.16	NS		0.22	0.15	NS	

NS: Non-significant; V: Variety; I: Inoculation level

The year had no significant impact on the yield parameters. Among the genotypes, the largest number of pods was noted for PSM 4, the longest pods for PSM 3, the highest number of seeds per pod for AP 1 and the heaviest seeds, resulting in the largest harvest index, for AP 3 in both years, whereas VL 7 performed poorly for most of the yield characters.

Table 3b
Effects of rhizobial inoculation on test weight and harvest index of vegetable pea

Genotype	2004–05				2005–06			
	0	20	40	Mean	0	20	40	Mean
				Test weight (g)				
Arkel	47.50	48.00	48.50	48.00	47.00	48.00	48.00	47.66
PSM 3	44.00	44.50	44.50	44.33	43.00	44.00	44.50	43.83
PSM 4	41.00	41.50	42.00	41.50	40.00	41.00	41.00	40.66
PMA 6	47.00	47.50	48.00	47.50	45.00	45.50	45.50	45.33
VL 7	42.00	42.50	43.00	42.50	41.00	41.50	42.00	41.50
AP 1	39.00	40.00	40.00	39.66	40.00	40.50	41.00	40.50
AP 3	46.00	47.00	47.50	46.83	46.00	46.50	46.50	46.33
NDVP 12	46.00	46.50	47.00	46.50	45.00	45.50	45.50	45.33
Mean	44.06	44.68	45.06		43.37	44.06	44.25	
	V	I	V × I		V	I	V × I	
SEM ±	0.642	0.393	1.112		0.554	0.339	0.960	
C.D. 5%	1.87	NS	NS		1.62	NS	NS	
				Harvest index (%)				
Arkel	38.84	40.00	40.20	39.68	35.12	38.70	38.80	37.54
PSM 3	35.71	38.13	39.50	37.78	34.54	37.70	37.65	36.96
PSM 4	34.48	35.93	36.20	35.53	35.00	37.70	37.70	36.80
PMA 6	29.72	32.63	33.10	31.81	29.47	31.30	31.35	30.70
VL 7	23.37	25.38	26.10	24.95	23.24	24.30	24.70	24.08
AP 1	31.48	33.45	33.75	32.89	33.84	34.40	34.45	34.23
AP 3	39.47	41.02	41.95	40.81	38.88	43.30	43.25	41.81
NDVP 12	33.33	33.95	34.05	33.71	30.00	31.25	31.45	30.90
Mean	33.30	35.06	35.60		32.51	34.83	34.91	
	V	I	V × I		V	I	V × I	
SEM ±	0.92	0.56	1.16		1.31	0.75	2.27	
C.D. 5%	2.70	1.68	NS		3.83	2.25	NS	

NS: Non-significant; V: Variety; I: Inoculation level

Nodulation characters

Seed inoculation of vegetable pea with *Rhizobium leguminosarum* improved the nodulation characters (Table 4). It enhanced the number of nodules per plant by 20.68–35.00% in 2004–05 and by 16.66–28.57% in 2005–06. Inoculation at 20 and 40 g kg^{-1} seed increased the number of nodules by 24.00 and 30.62% in the first year and by 20.62 and 26.21% in the second year compared to no inoculation. This was reflected in the improved nodule dry weight per plant, with a response range of 5.00–9.48% in 2004–05 and 5.79–9.82% in 2005–06. Increases in nodule dry weight per plant of 5.59 and 7.25% were observed at the 20 and 40 g kg^{-1} levels of seed inoculation in the first year and 6.26 and 8.79%, respectively, in the second year. The increased number and dry weight of nodules after inoculation also enhanced the root dry weight of the vegetable pea genotypes. The increase ranged from 7.22–12.83% in the first year and from 5.81–15.54% in the second year over no inoculation. Compared to the control, increases of 7.88 and 10.56% in the first year and 9.30 and 12.14% in the second year were recorded after inoculation with 20 and 40 g kg^{-1} seed, respectively.

The large variation in nodulation is an indication of genotypic differences and can be used to improve atmospheric nitrogen fixation. Srivastava and Ahlawat (1995) also reported enhanced nodulation in terms of number of nodules per plant and their dry weight in garden pea after seed inoculation with *Rhizobium.*

Table 4
Effects of rhizobial inoculation on nodulation parameters of vegetable pea

Genotype	2004–05				2005–06			
	0	20	40	Mean	0	20	40	Mean
				Number of nodules/plant				
Arkel	26.00	32.50	34.50	31.00	25.00	31.00	32.50	29.50
PSM 3	29.00	35.00	37.00	33.66	27.00	32.00	33.50	30.83
PSM 4	31.00	39.00	40.00	36.66	30.00	37.50	39.50	35.66
PMA 6	23.00	29.00	32.00	28.00	21.00	26.00	28.00	25.00
VL 7	19.00	23.00	25.00	22.33	20.00	24.00	25.50	23.16
AP 1	29.00	35.00	35.50	33.16	30.00	36.50	37.00	34.50
AP 3	25.00	33.00	34.50	30.83	27.00	31.00	32.00	30.00
NDVP 12	22.00	26.50	28.50	25.66	26.00	30.50	32.00	29.50
Mean	25.50	31.62	33.37		25.75	31.06	32.50	
	V	I	V × I		V	I	V × I	
SEM ±	0.68	0.41	1.18		0.64	0.39	1.11	
C.D. 5%	2.00	1.22	NS		1.88	1.15	NS	
				Nodule dry weight (mg/plant)				
Arkel	69.50	74.00	75.00	72.83	69.00	72.00	74.00	71.66
PSM 3	70.00	73.00	75.00	72.66	67.00	71.00	72.00	70.00
PSM 4	74.00	78.00	80.00	77.33	70.00	75.00	77.00	74.00
PMA 6	62.00	66.00	67.00	65.00	56.00	61.00	62.00	59.66
VL 7	58.00	63.00	64.00	61.66	52.00	55.50	57.50	55.00
AP 1	72.00	76.00	76.50	74.83	68.00	71.50	73.50	71.00
AP 3	70.00	73.00	74.00	72.33	66.00	70.00	72.00	69.33
NDVP 12	69.00	72.00	73.00	71.33	63.00	67.00	68.00	66.00
Mean	68.06	71.87	73.06		63.87	67.87	69.50	
	V	I	V × I		V	I	V × I	
SEM ±	0.65	0.40	1.13		1.14	0.69	1.97	
C.D. 5%	1.91	1.17	NS		3.34	2.04	NS	
				Root dry weight (mg/plant)				
Arkel	410.00	440.00	445.00	431.66	380.00	430.00	435.00	415.00
PSM 3	450.00	480.00	490.00	473.33	430.00	450.00	460.00	446.66
PSM 4	460.00	500.00	510.00	490.00	450.00	480.00	490.00	473.33
PMA 6	390.00	430.00	445.00	421.66	360.00	390.00	405.00	385.00
VL 7	370.00	410.00	425.00	401.66	350.00	390.00	405.00	381.66
AP 1	455.00	485.00	495.00	478.33	435.00	475.00	485.00	465.00
AP 3	415.00	440.00	450.00	435.00	395.00	430.00	440.00	421.66
NDVP 12	410.00	440.00	455.00	435.00	370.00	420.00	435.00	408.33
Mean	420.00	453.12	464.37		396.25	433.12	444.37	
	V	I	V× I		V	I	V × I	
SEM ±	9.83	6.02	17.03		10.68	6.54	18.50	
C.D. 5%	28.77	17.61	NS		31.25	19.13	NS	

NS: Non-significant; V: Variety; I: Inoculation level

Nodulation was slightly better during 2004–05 than in 2005–06. The genotype PSM 4 had the highest number of nodules, leading to greater nodule and root dry weights per plant, while nodulation was the lowest in VL 7 in both years.

Table 5

Effects of rhizobial inoculation on biochemical parameters of vegetable pea

Genotype	2004–05				2005–06			
	0	20	40	Mean	0	20	40	Mean
				Seed nitrogen (%)				
Arkel	3.55	3.85	3.92	3.77	3.45	3.75	3.80	3.66
PSM 3	3.60	3.85	3.90	3.78	3.45	3.72	3.77	3.64
PSM 4	3.40	3.65	3.72	3.59	3.50	3.77	3.82	3.70
PMA 6	3.25	3.52	3.58	3.45	3.15	3.45	3.50	3.36
VL 7	3.20	3.47	3.52	3.40	3.05	3.45	3.50	3.33
AP 1	3.45	3.75	3.80	3.66	3.55	3.87	3.92	3.78
AP 3	3.50	3.72	3.77	3.66	3.40	3.77	3.82	3.66
NDVP 12	3.60	3.87	3.97	3.81	3.47	3.75	3.82	3.68
Mean	3.44	3.71	3.77		3.37	3.69	3.74	
	V	I	V × I		V	I	V× I	
SEM ±	0.044	0.027	0.076		0.026	0.020	0.046	
C.D. 5%	0.130	0.079	NS		0.078	0.060	NS	
				Seed protein (%)				
Arkel	21.87	24.05	24.53	23.48	21.56	23.43	23.75	22.91
PSM 3	22.49	24.05	24.37	23.64	21.56	23.28	23.56	22.80
PSM 4	21.24	22.81	23.28	22.44	21.87	23.59	23.90	23.12
PMA 6	20.31	22.02	22.31	21.54	19.68	21.56	21.87	21.03
VL 7	19.99	21.71	22.03	21.24	19.06	21.55	21.87	20.82
AP 1	21.56	23.43	23.74	22.91	22.18	24.21	24.52	23.64
AP 3	21.87	23.28	23.59	22.91	21.24	23.59	23.90	22.91
NDVP 12	22.49	24.21	24.84	23.85	21.68	23.43	23.87	22.99
Mean	21.48	23.19	23.58		21.10	23.08	23.40	
	V	I	V × I		V	I	V× I	
SEM ±	0.271	0.166	0.467		0.173	0.116	0.299	
C.D. 5%	0.793	0.485	NS		0.506	0.348	NS	
				Nitrogenase activity (μM C_2H_4/plant/h)				
Arkel	2.80	3.15	3.25	3.06	2.90	3.30	3.40	3.20
PSM 3	2.80	3.30	3.50	3.20	2.90	3.50	3.60	3.33
PSM 4	3.10	3.60	3.70	3.46	3.20	3.70	3.80	3.56
PMA 6	2.70	3.30	3.40	3.13	2.80	3.10	3.20	3.03
VL 7	2.70	3.10	3.25	3.01	2.80	3.10	3.20	3.03
AP 1	2.90	3.30	3.40	3.20	2.80	3.40	3.50	3.23
AP 3	2.90	3.20	3.30	3.13	2.90	3.30	3.40	3.20
NDVP 12	2.80	3.30	3.40	3.16	2.80	3.10	3.15	3.01
Mean	2.83	3.28	3.40		2.88	3.31	3.40	
	V	I	V × I		V	I	V × I	
SEM ±	0.057	0.044	0.099		0.054	0.034	0.093	
C.D. 5%	0.167	0.132	NS		0.215	0.107	NS	

NS: Non-significant; V: Variety; I: Inoculation level

Biochemical characters

The rhizobial inoculation of the seeds of vegetable pea had a beneficial effect on the biochemical characters (Table 5). It improved the nitrogen (6–14%) and protein concentration (7–11%) in the pea seeds, as well as enhancing the nitrogenase activity (11–24%) in the root nodules owing to its role in photosynthesis and in energy transformation processes. A conspicuous increase in the concentration and uptake of nitrogen in pea owing to inoculation was also observed by Srivastava and Ahlawat (1995). Sibbal et al. (2002) also found a significant increase in nitrogenase activity after inoculating seeds with *Rhizobium leguminosarum* as compared to no inoculation. The year had little influence on any of the biochemical parameters. Among the genotypes, the seeds of NDVP 12 were rich in nitrogen and protein, while those of VL 7 were poor for these characters; the highest nitrogenase activity was observed in PSM 4 and the lowest in VL 7 in both years.

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EFFECT OF LIQUID AND CYST FORMULATIONS OF *Azospirillum* WITH INORGANIC NITROGEN ON THE GROWTH AND YIELD OF RICE

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The nitrogen-fixing rhizobacterium *Azospirillum* lives in close association with plant roots, where it exerts beneficial effects on the plant growth and yield of many crops of agronomic importance. As carrier-based inoculants have a short shelf life and poor quality, new liquid and cyst formulations of inoculants have been developed and standardized for *Azospirillum*. In the present investigation, experiments were conducted to study the effect of liquid and cyst formulations of *Azospirillum*, combined with inorganic nitrogen, on the growth and yield of rice. Inoculation with the cyst formulation of *Azospirillum* enhanced the plant height, biomass and N uptake of the plants, the available nitrogen content of the soil and the yield of rice to the greatest extent when compared to carrier-based *Azospirillum*, followed by the liquid formulation. The results of the present study clearly indicated that the cyst and liquid formulations of *Azospirillum* could be used as bioinoculants more effectively than the carrier-based one.

Key words: *Azospirillum*, liquid and cyst formulation, rice growth and yield

Introduction

Among the free-living, nitrogen-fixing bacteria, *Azospirillum* is considered to have more efficient nitrogenase properties than other nitrogen fixers (Okon, 1985). These organisms are found in abundant numbers in the rhizosphere as well as in the intercellular spaces of the roots of certain cereals and other plants (Bashan and Holguin, 1997). Some of the suggested modes of action for *Azospirillum* are the secretion of phytohormones, nitrogen fixation, the production of undefined signal molecules that can interfere with the plant metabolism, nitrite production and the enhancement of mineral uptake by plants (Okon and Itzigsohn, 1995). They thus exert a beneficial effect on the growth of plants and increase the yield of many crops of agronomic importance (Okon and Vanderleyden, 1997).

Azospirillum species have probably been studied to the greatest extent and appeared to have significant potential for commercial application. FAO (1991) reported that most of the international producers of biofertilizers are engaged in the production of carrier-based inoculants, which generally have short shelf life and poor quality. A frequent observation is that in peat-based inoculants, the number of viable cells decreases from 10^9 to 10^7 cfu per g after 90 days of storage (Okon and Itzigsohn, 1995). The inoculant formulation has a crucial effect on the inoculation process because the chosen formulation determines the potential success of the inoculant. There has been limited development of inoculants involving *Azospirillum* in recent years. Hence, as an alternative to the carrier-based option, new liquid and cyst formulations of inoculants have been developed and standardized for *Azospirillum* (Thamizh Vendan and Thangaraju, 2006; 2007a, b). In the present investigation, experiments were conducted to study the effect of liquid and cyst formulations of *Azospirillum* combined with inorganic nitrogen on the growth and yield of rice.

Materials and methods

Bacterial culture, media and culture conditions

The standard *Azospirillum lipoferum* strain *Az*-204, maintained in N-free malic acid medium (Dobereiner and Day, 1974), was used for this study. Culture maintenance, mass production and liquid bioinoculant preparations were done in N-free malic acid medium. The *Azospirillum* culture was grown in an environmentally controlled shaker at 100 rpm at 30°C.

The liquid (Thamizh Vendan and Thangaraju, 2006) and cyst (Thamizh Vendan and Thangaraju, 2007a) formulations developed and standardized earlier were used in this study. The dosage of the formulations for various inoculation methods was also standardized (Thamizh Vendan and Thangaraju, 2007b). To evaluate the performance of the liquid and cyst formulations of *Azospirillum*, field experiments were laid out with the rice variety ADT-46 (short duration, 115 days). Inoculation was carried out using all three recommended methods, namely seed treatment, seedling dipping and soil application.

Inoculation with Azospirillum *through seed treatment*

The rice seeds (ADT-46) were first surface-sterilized with 0.5% hydrogen peroxide for one min, followed by 80% alcohol for one min. The seeds were then washed several times with sterile distilled water to remove excess chemicals. The surface-sterilized rice seeds were inoculated with the standardized quantity (10 ml kg^{-1} of seed) of the liquid and cyst formulations of *Azospirillum* and an equal volume of sterile water. The carrier (lignite)-based inoculum (as control) was applied at 20 g per kg of seed and sterile water was added to make a slurry (50 ml per kg of seed and the inoculated seeds were shade dried for 30 min. The inoculated seeds were sown in a mat nursery filled with sterilized soil.

Inoculation with Azospirillum *through seedling dipping*

After 25 days, the seedlings were carefully removed from the nursery and the roots were washed thoroughly in sterile water. The roots of the seedlings were dipped in the standardized quantity (150 ml ha^{-1}) of liquid or cyst *Azospirillum* inoculum with 25 litres of sterile water. One kg of the carrier-based formulation was mixed with 25 litres of sterile water to make a slurry and the seedlings required for one ha (seedlings emerged from 8 kg seed) were dipped in the slurry for the control plot and kept in the shade. After 30 min dipping, the inoculated seedlings were transplanted.

Inoculation with Azospirillum *through soil application*

Soil application with the liquid and cyst formulations of *Azospirillum* was done on the 10th day after transplanting (DAT). The standardized quantity of inoculum (300 ml ha^{-1}) was applied with 25 kg of well-powdered farmyard manure. Carrier-based *Azospirillum* was inoculated at 2 kg ha^{-1} with 25 kg of well-powdered farmyard manure.

Design and treatments

The fertilizers were applied @ 50 : 50 PK kg ha^{-1} and the nitrogenous fertilizer was applied at graded levels as given below. The experiments were conducted in a factorial random block design, replicated thrice, with the following treatment schedule:

Factor I: *Azospirillum lipoferum* formulation	Factor II: Nitrogen levels
T_1 – Control	N_1 – 0% N kg/ha (0 kg N/ha)
T_2 – Carrier-based *Azospirillum*	N_2 – 75% N kg/ha (93.75 kg N/ha)
T_3 – Liquid formulation of *Azospirillum*	N_3 – 100% N kg/ha (125 kg N/ha)
T_4 – Cyst formulation of *Azospirillum*	

Biometric and yield measurements

The biometric observations were recorded at monthly intervals and yield parameters were recorded at the time of harvest. The plant height was measured from the bottom of the root to the tip of the plant and expressed in cm. The weight of the dry plant samples was recorded and expressed as g $plant^{-1}$. The total nitrogen content of the plant was estimated by the Kjeldahl method (Humphries, 1956). The N uptake was derived from total N content and plant biomass and expressed as mg/plant. The available nitrogen content in the soil was estimated by the alkaline permanganate method suggested by Subbiah and Asija (1956). The grain yield of rice was recorded and expressed as t ha^{-1}. All the data obtained from the above experiments were subjected to factorial RBD statistical analysis (Panse and Sukhatme, 1985).

Results and discussion

The nitrogen-fixing rhizobacterium *Azospirillum* lives in close association with plant roots, where it exerts beneficial effects on the plant growth and yield of many crops of agronomic importance (Okon and Labandera-Gonzalez, 1994; Okon and Vanderleyden, 1997). In the present investigation, experiments were conducted to evaluate the performance of liquid and cyst formulations of *Azospirillum*, combined with graded levels of nitrogen on rice. A gradual increase in the plant height was observed from 30 DAT to 120 DAT. The cyst formulation of *Azospirillum* enhanced the height of rice plants to a maximum of 90.1 cm at 120 DAT, followed by the liquid formulation (88.2 cm) and carrier-based *Azospirillum* (83.9 cm). The minimum height (73.9 cm) was recorded in the uninoculated control treatment (Fig. 1). Among the N levels, all the formulations of *Azospirillum* performed better at the 75% N level, when compared to the 0 and 100% levels. Treatments inoculated with carrier-based *Azospirillum* resulted in the lowest biomass production. The cyst formulation of *Azospirillum* increased the biomass more effectively than the liquid formulation. Among the N levels, 75% N level proved superior to the other N levels (Fig. 2).

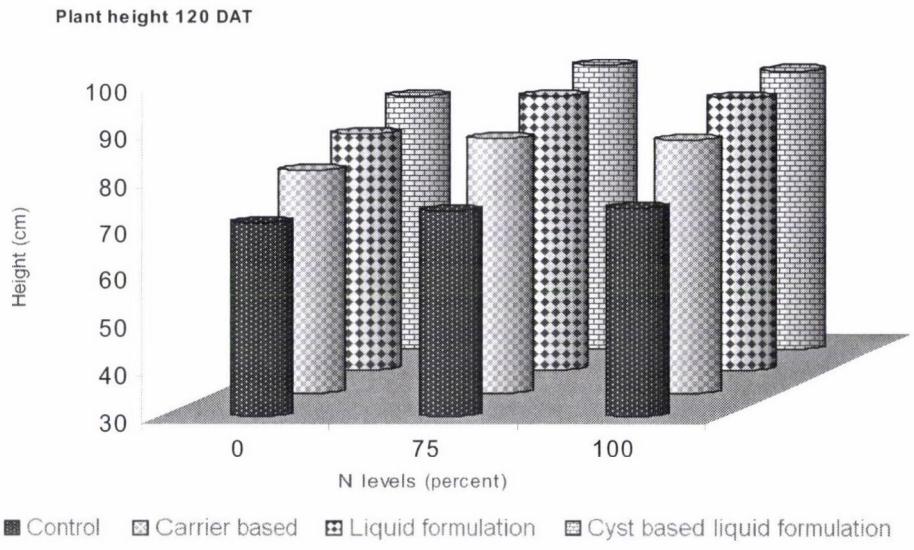

Fig. 1. Effect of liquid and cyst formulations of *Azospirillum*, combined with graded levels of N, on the plant height of rice

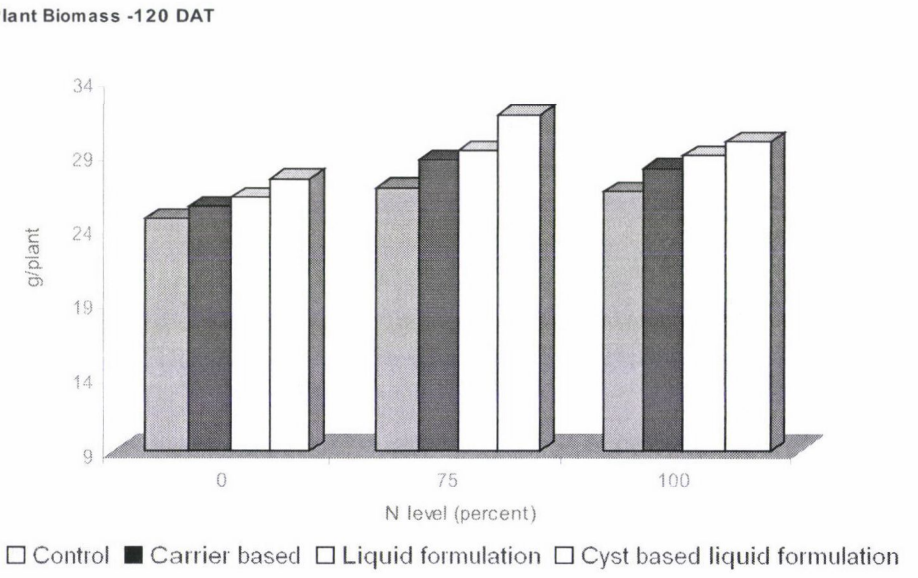

Fig. 2. Effect of liquid and cyst formulations of *Azospirillum*, combined with graded levels of N, on the plant biomass of rice

An increase in the height of crop plants due to inoculation with *Azospirillum* has been reported by several workers (El-Nagger and Mohamaud, 1994; Hemavathi, 1997). This was probably caused by increased cell elongation and multiplication due to enhanced nutrient uptake by plants following

inoculation with *Azospirillum* as well as application of nitrogen fertilizer. The plant growth-promoting substances (IAA and GA) secreted by *Azospirillum* might play an important role in root elongation and shoot growth (Tien et al., 1979). In general, *Azospirillum* inoculation enhanced the proliferation of the root system, which in turn accelerated mineral uptake and consequently increased the biomass (Okon, 1985; Sattar and Gaur, 1987). An increase in maize and sorghum biomass was observed in greenhouse and field experiments by Dobbelaere et al. (2001). The prolonged survival of the cyst formulation in the rhizosphere region might be the reason for the better performance compared with carrier-based *Azospirillum* inoculation.

The total N content of rice was estimated from 30 DAT to 120 DAT and the highest value was recorded at 60 DAT, after which it gradually decreased. In rice the maximum N content of 1.31% was recorded in the treatment inoculated with the cyst formulation of *Azospirillum* combined with the 75% N level (Table 1). The lowest N content was recorded in the uninoculated treatments in all the sampling periods when compared to inoculation with *Azospirillum*. Among the N levels, 75% and 100% were statistically on par with each other. The cyst formulation of *Azospirillum* combined with 75% N resulted in the highest N uptake when compared to the other treatments, while the uninoculated control had the lowest N uptake in all the sampling periods (Fig. 3). The uptake of N was found to increase up to 60 DAT, with a slight decline at 90 DAT. However, maximum nitrogen uptake was recorded in all the treatments at 120 DAT. Data on the available N content of the rhizosphere clearly showed that inoculation with *Azospirillum*, irrespective of the formulation, resulted in the enhancement of the available N content (Table 2). The cyst formulation of *Azospirillum* combined with the 75% N level led to the highest available N content (135.2 kg/ha) in the rice rhizosphere, whereas 119.6 kg/ha was recorded in the respective control at 90 DAT.

Table 1

Effect of liquid and cyst formulations of *Azospirillum*, combined with graded levels of N, on the total nitrogen content (kg/ha) of rice

Treatments	30 DAT			60 DAT			90 DAT			120 DAT		
N level (%)	0	75	100	0	75	100	0	75	100	0	75	100
T_1	0.61	0.80	0.88	0.87	1.05	1.12	0.73	0.94	0.85	0.67	0.86	0.80
T_2	0.74	0.89	0.95	0.94	1.14	1.18	0.81	0.97	0.94	0.78	0.89	0.86
T_3	0.87	0.98	1.02	1.03	1.22	1.20	0.84	0.99	0.96	0.82	0.91	0.88
T_4	0.91	1.12	1.10	1.12	1.31	1.28	0.87	1.03	0.98	0.85	0.95	0.91
Mean	0.78	0.94	0.98	0.99	1.18	1.19	0.81	0.98	0.93	0.78	0.90	0.86
	SEd		$CD_{(0.05)}$	SEd		$CD_{(0.05)}$	SEd		$CD_{(0.05)}$	SEd		$CD_{(0.05)}$
Treatment	0.016		0.033	0.006		0.014	0.009		0.020	0.007		0.014
N level	0.014		0.029	0.005		0.012	0.008		0.017	0.004		0.008
Interaction	0.028		0.058	0.011		0.024	0.017		0.035	0.019		0.039

DAT: Days after transplanting; N% level: 75% (93.75 kg N/ha); 100% (125 kg N/ha); T_1: Uninoculated control; T_2: Carrier (lignite)-based *Azospirillum*; T_3: Liquid formulation of *Azospirillum*; T_4: Cyst formulation of *Azospirillum*

Table 2

Effect of liquid and cyst formulations of *Azospirillum*, combined with graded levels of N, on the available nitrogen content (kg/ha) in the rice rhizosphere soil

Treatments	30 DAT			60 DAT			90 DAT			120 DAT		
N level (%)	0	75	100	0	75	100	0	75	100	0	75	100
T_1	95.2	99.8	103.0	97.8	112.0	118.2	98.6	119.6	120.4	88.4	98.8	101.4
T_2	108.2	119.4	122.0	118.4	126.8	125.6	119.8	128.0	127.6	92.6	95.2	95.0
T_3	112.0	121.8	122.8	123.3	131.6	130.8	123.8	132.4	132.0	94.0	95.8	95.0
T_4	114.4	122.8	124.0	124.6	134.8	132.6	125.0	135.2	134.8	95.6	96.2	95.6
Mean	107.4	116.0	117.4	116.0	126.3	126.8	116.8	128.8	128.7	92.6	96.5	96.7
	SEd		$CD_{(0.05)}$	SEd		$CD_{(0.05)}$	SEd		$CD_{(0.05)}$	SEd		$CD_{(0.05)}$
Treatment	0.524		1.087	0.384		0.798	0.671		1.393	0.431		0.894
N level	0.453		0.941	0.333		0.691	0.581		1.206	0.373		0.774
Interaction	0.907		1.882	0.666		1.382	1.163		2.413	0.747		1.549

DAT: Days after transplanting; N% level: 75% (93.75 kg N/ha); 100% (125 kg N/ha); T_1: Uninoculated control; T_2: Carrier (lignite)-based *Azospirillum*; T_3: Liquid formulation of *Azospirillum*; T_4: Cyst formulation of *Azospirillum*

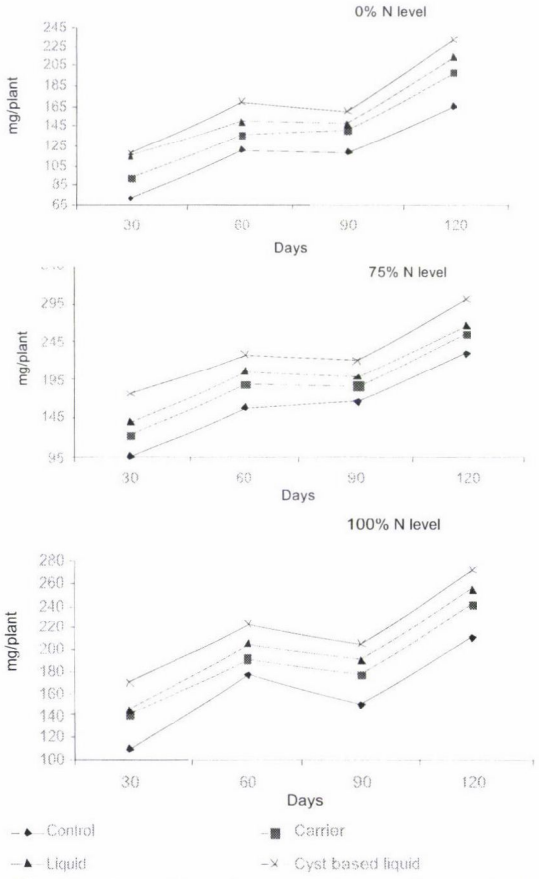

Fig. 3. Effect of liquid and cyst formulations of *Azospirillum* on the N uptake of rice at graded levels of N

Since plants inoculated with *Azospirillum* had high N content, it is reasonable to think that inoculation may have enhanced plant N uptake by increasing the availability of N in the rhizosphere due to the activity of the bacteria. The present result is in agreement with Rao and Rao (1983), who found increased total nitrogen content in paddy due to *Azospirillum* inoculation. The N uptake of rice was found to be increased by the inoculation of *Azospirillum* compared to the N control treatment. The data showed that the uptake of nitrogen was maximum during active tillering and between the milky and maturity stages of rice. The growth hormones secreted by *Azospirillum* enhanced the proliferation of the root system, which in turn increased the uptake of nitrogen, as reported by Okon and Kapulnik (1986) and Sattar and Gaur (1987). The enhanced root system and increased rhizosphere availability may have enhanced the N uptake in plants inoculated with *Azospirillum*. Saubidet et al. (2002) found an increase in the N uptake in wheat plants inoculated with *Azospirillum* sp. and concluded that increased N uptake is the mechanism of plant growth promotion in these plants. Similarly, the increased population and prolonged survival of *Azospirillum* in the inoculated soil enhanced the fixation of nitrogen and consequently the available N content was increased. This is in line with Thamizh Vendan and Subramanian (1999), who found increased available N content in the *Azospirillum*-inoculated paddy rhizosphere with low levels of nitrogenous fertilizers.

The highest grain yield of 7.88 t/ha was recorded due to inoculation with the cyst formulation of *Azospirillum* combined with the 75% N level, followed by the liquid formulation of *Azospirillum* with the 75% N level (7.80 t/ha). The minimum grain yield of 6.27 t/ha was recorded in the uninoculated control at the 0% N kg/ha level (Table 3). Many scientists have reported that the response of rice to *Azospirillum* inoculation was more pronounced at lower levels of fertilizer nitrogen than at higher levels. As nitrogen inhibits the nitrogenase enzyme, inoculation with *Azospirillum* may contribute to yield increases particularly when plant access to mineral nitrogen in the soil is restricted. Upon *Azospirillum* inoculation an alteration in root morphology was observed, which has been ascribed to the bacterial production of plant growth-regulating substances (Kapulnik et al., 1985; Fallik et al., 1994). Higher nutrient uptake by inoculated roots and the improved water status of the plant could, in turn, be the main factors enhancing plant growth and yield (Okon, 1994).

In the present study, the cyst and liquid formulations of *Azospirillum* performed well when compared to the carrier-based one. A large number of factors, such as easy adherence to the roots, increased population, prolonged survival in the rhizosphere, and enhanced cell tolerance to desiccation and temperature stress, might have attributed to the superior performance of the cyst and liquid formulations over carrier (lignite)-based *Azospirillum*. A similar result was obtained by Singleton et al. (2002), who developed a liquid formulation of *Rhizobium* and found increased soybean yield compared with the peat-based carrier inoculant.

Table 3
Effect of liquid and cyst formulations of *Azospirillum*, combined with graded levels of N, on the grain yield of rice

Treatments	Grain yield (t/ha)			
N % level	0	75	100	Mean
T_1	6.27	6.43	6.54	6.41
T_2	7.21	7.54	7.51	7.42
T_3	7.34	7.80	7.64	7.60
T_4	7.36	7.88	7.74	7.66
Mean	7.05	7.42	7.36	7.27
		SEd		CD (0.05)
Treatment		0.250		0.518
N level		0.216		0.449
Interaction		0.433		0.898

N% level: 75% (93.75 kg N/ha); 100% (125 kg N/ha); T_1: Uninoculated control; T_2: Carrier (lignite)-based *Azospirillum*; T_3: Liquid formulation of *Azospirillum*; T_4: Cyst formulation of *Azospirillum*

The results of the present study clearly indicated that inoculation with the cyst and liquid formulations of *Azospirillum* enhanced the growth and yield of rice when compared to the carrier-based formulation.

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EFFECT OF CHEMICAL COMPOSITION OF SUGAR SORGHUM AND THE CULTIVATION TECHNOLOGY ON ITS UTILISATION FOR SILAGE PRODUCTION

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The objective of this research project was to determine the chemical composition of sugar sorghum (*Sorghum saccharatum*) and the suitability of sorghum forage for ensiling, and to recommend an optimal cultivation technology in order to obtain raw material for the production of high quality silage. The object used for comparison was a medium late cultivar of *Zea mays,* Magister (FAO 270), characterised as the "stay green" type, which is desirable if good quality forage and silage is to be obtained under the conditions prevailing in Poland. The experiment was set up in the years 2004–2005 on a soil suitable for the cultivation of these crops. The experimental material consisted of four cultivation combinations chosen on the basis of our current knowledge on the subject. The results show sugar sorghum to be an interesting fodder plant, characterised by favourable biological and chemical properties. Sorghum cultivation provides nutritionally valuable forage and important silage material. Irrespective of the experimental combination employed, the sorghum plants gave good quality silage, as confirmed by their chemical composition and digestibility. Sugar sorghum should be treated as a fodder grass complementary to maize. The different stem morphological structures of sorghum and maize may cause differences in the quality assessment of the silage obtained from the two crops. The various cultivation treatments failed to diversify the chemical composition of the sorghum forage and silage. Therefore, the mode of cultivation is of secondary importance and the decisive factor is the yield of the aboveground parts.

Key words: *Sorghum saccharatum,* optimisation of cultivation, chemical properties, mixed sowing

Introduction

Species from the sorghum genus are interesting cereal and fodder grasses characteristic of the warm, dry climate zone and are highly valued in many countries of Africa, Asia and South America, while also becoming increasingly popular in Europe. One of the more expansionary species of this genus is sugar

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sorghum [*Sorghum saccharatum* (L.) Pers.]. Among the advantages of this species as a fodder plant is its high fresh matter yield and considerable resistance to drought. For these reasons, sugar sorghum is beginning to be perceived as being competitive with maize. Its high sugar content means that the forage obtained from sorghum can provide valuable raw material for ensiling. Sorghum forage, like maize, is usually fed to animals after ensiling. Therefore, it is important to assess the suitability of this species for ensiling, its chemical composition, and its cultivation potential under the climatic and soil conditions of Poland.

Bochniarz (1969) claims that, if an appropriate cultivar is cultivated with a suitable technology, sorghum could become a valuable fodder plant in Poland. Alongside its cultivation in pure stands, sorghum could also be cultivated in mixtures with other grass species, especially with maize.

The objective of this research project was to analyse the chemical composition of sugar sorghum, to determine the suitability of sorghum forage for ensiling and to recommend the optimal crop combination in order to obtain raw material for the production of high quality silage.

Materials and methods

Experiments were initiated in 2004 to compare *Sorghum saccharatum* (L.) Pers., cultivar Sucrosorgo 506, with Magister (FAO 270), a medium late cultivar of *Zea mays,* with the "stay green" character which is desirable if good quality forage and silage are to be obtained under Polish conditions. It is worth mentioning here that sorghum and maize are both grasses of the C-4 type, with similar site requirements and end uses. The experiments were established on soil suitable for the cultivation of these grasses, with a humus content of approx. 1.22%, silt and clay fraction 16%, pH_{KCl} = 5.5, and containing 8.3 mg P_2O_5, 14.3 mg K_2O and 5.8 mg MgO per 100 g soil.

Cultivation treatments

The experiment comprised five cultivation treatments, each on an area of 112 m^2, reflecting current knowledge on sorghum and its cultivation technology.

Treatment A: Cultivation of sorghum in a pure stand. Density: 180,000 plants per ha. Inter-row spacing: 70 cm, in-row spacing: 7 cm.

Treatment B: Cultivation of maize in a pure stand. Density: 90,000 plants per ha. Inter-row spacing: 70 cm, in-row spacing: 15 cm.

Treatment C: Cultivation of alternate rows of sorghum and maize in a mixed stand. Density: 90,000 sorghum plants and 40,000 maize plants per ha. Inter-row spacing: 70 cm, in-row spacing: sorghum – 7 cm, maize – 17 cm.

Treatment D: Mixed stand of sorghum and maize with a row combination of sorghum-sorghum-maize. Density: 130,000 sorghum plants and 25,000 maize plants per ha. Inter-row spacing: 70 cm, in-row spacing: sorghum – 7 cm, maize – 17 cm.

Treatment E: Mixed stand with sorghum plants sown 12 cm from the maize rows. Density: 180,000 sorghum plants and 55,000 maize plants per ha. Inter-row spacing of maize: 70 cm, in-row spacing: maize – 26 cm, sorghum – 7 cm.

Sowing was carried out in the last third of April using a Monosem drill equipped with a disk. The following fertilisation doses were applied: N – 160 kg, P – 80 kg, K – 170 kg/ha.

Forage assessment

The assessment of the experimental forages comprised a wide spectrum of chemical properties as well as the aboveground yield of the sorghum and maize plants. The analytical studies included the determination of crude protein content (Kjeldahl method), soluble sugars (Dubois et al., 1956), cellulose and lignin (Van Soest and Wine, 1968), hemicelluloses (Heyland, 1959), acid detergent fibre (ADF) and neutral detergent fibre (NDF), nitrate nitrogen (Johnson and Ulrich, 1950) and carotene dyes. Attention was also paid to cyanogenic glucosides and plant mineral composition. The concentrations of phosphorus and magnesium were determined using the colorimetric method, calcium by the titration-precipitation method, potassium and sodium by flame photometry and silica by the gravimetric method.

Silage assessment

Plants for ensiling were harvested when sorghum reached the stage of kernel milk maturity and maize the stage of dough maturity. The plant material harvested from individual experimental treatments was ensiled in polyethylene microsilos 10 cm in diameter and 50 cm in height. Each microsilo was filled with 3 kg of cut, compacted forage and then sealed with a rubber cork equipped with a wine tube. There were four microsilos for each treatment. No silage additives were used. The microsilos were opened after 90 days and samples for chemical analyses and for *in vitro* studies were collected. The following parameters were determined: pH using an Elmetron pH-meter, dry matter, crude protein and crude ash according to AOAC (2000), reducing sugars and starch according to the method of McDonald and Henderson (1964), crude fibre according to the Henneberg and Stohman method, ADF and NDF, according to Goering and Van Soest and organic acids (acetic, propionic, butyric and lactic acids) by gas chromatography using the Varion Star 3400 CX apparatus.

In vitro studies took place in a Rusitec apparatus (Czerkawski and Breckenridge, 1997) equipped with four fermenters, so the following four silages were used: one silage made of maize (treatment B) and three mixtures of maize and sorghum (treatments C, D and E). The sorghum silage was not evaluated, since the main purpose of the experiments was to assess mixtures of maize and sorghum.

Each of the four fermenters (1 L in volume) was filled with 100 ml pre-warmed buffer solution (artificial saliva: Czerkawski and Breckenridge, 1997) and 900 ml strained rumen fluid collected from a rumen-fistulated cow. The nylon bags (70 × 130 mm) had a pore size of 100 μm as recommended by Carro et al. (1995). The average daily feed supply was 11 g of dry matter (DM). The turnover rate of the liquid was 500 ml d^{-1}. Each 10-day experimental period consisted of 4 days of adaptation of the rumen microbes to the system and diet and 6 days of sample collection. During the last 5 days of the experiment, samples were taken every day.

The pH, ammonium and volatile fatty acids in the rumen juice after incubation were determined using the same methods employed to analyse silage, as were the dry matter, crude protein, crude fibre, ADF and NDF in the samples after the incubation. The digestibility of dry matter, crude protein, crude fibre, ADF and NDF was calculated on the basis of the difference in the contents of these constituents in the samples before and after incubation in the Rusitec apparatus.

Results

Forage chemical composition

The quality analysis of *Sorghum saccharatum* included the organic and mineral constituents in the shoot organs and the chemical composition of the forage intended for ensiling from the different treatments.

The chemical composition of the leaf blades (Table 1) showed that sorghum is characterised by a high protein content and a lower level of structural carbohydrates than in *Zea mays*. The quantity of mineral constituents is close to the optimal value. However, the leaf blades accumulate considerable quantities

of nitrate nitrogen and cyanogenic glucosides. Thus, based on leaf quality, sorghum forage is more valuable than maize forage, but involves certain hazards. In the case of stem chemical composition (Table 2), a dominant organ due to its weight, it is noteworthy that the sugar content is nearly 70% higher in sorghum than in maize. Table 3 shows the chemical composition of cut shoots of sugar sorghum and maize used as raw material for ensiling. It is clear from the table that the differences between the species decreased markedly, no doubt as the result of differences in the morphological shoot structure.

Table 1
Chemical composition of leaf blades of sorghum and maize (g kg^{-1} DM)

Component	*Sorghum saccharatum*	*Zea mays*	$LSD_{0.05}$
Crude protein	179.32	133.12	15.365
Sugars	47.24	56.56	4.236
Cellulose	234.36	252.47	1.063
Hemicelluloses	199.34	227.39	7.164
Lignins	24.82	23.17	n.s.
Acid detergent fibre	259.18	275.53	10.131
Neutral detergent fibre	458.47	502.84	14.761
Crude ash	71.06	74.71	2.066
K	18.53	19.23	n.s.
Ca	12.05	10.64	n.s.
Mg	3.16	2.16	0.363
P	2.45	1.01	0.471
Na	0.70	0.99	0.176
Si	3.29	6.50	1.833
β-carotene (mg g^{-1} DM)	0.533	0.4431	3.657
$N\text{-}NO_3$	1.14	0.28	0.379
HCN	++	–	–

++: high content; – : absent; n.s.: non-significant

Table 2
Chemical composition of stems of sorghum and maize (g kg^{-1} DM)

Component	*Sorghum saccharatum*	*Zea mays*	$LSD_{0.05}$
Crude protein	86.25	79.35	3.275
Sugars	142.61	84.12	10.363
Cellulose	276.23	269.83	n.s.
Hemicelluloses	218.05	193.64	n.s.
Lignins	25.91	27.42	n.s.
Acid detergent fibre	302.15	297.25	n.s.
Neutral detergent fibre	520.13	490.82	n.s.
Crude ash	36.01	56.74	10.338
K	16.42	23.86	1.738
Ca	3.07	5.67	n.s.
Mg	1.90	1.38	0.463
P	1.16	0.32	0.391
Na	2.13	1.16	0.255
Si	0.25	4.16	0.591
β-carotene (mg g^{-1} DM)			
$N\text{-}NO_3$	3.92	2.59	0.196
HCN	–	–	–

– : absent; n.s.: non-significant

Table 3
Chemical composition of cut sorghum and maize shoots used as raw material for ensiling (g kg^{-1} DM)

Component	Treatment A	Treatment B	$LSD_{0.05}$
Crude protein	96.23	91.58	n.s.
Sugars	37.82	21.35	0.626
Cellulose	313.94	218.20	19.191
Hemicelluloses	241.42	255.73	n.s.
Lignins	39.93	22.04	4.199
Acid detergent fibre	352.92	240.24	18.863
Neutral detergent fibre	594.33	495.95	15.197
Crude ash	50.73	44.34	2.739
K	19.02	18.33	n.s.
Ca	6.81	8.68	0.717
Mg	2.94	1.39	0.496
P	1.68	2.17	0.372
Na	1.62	0.55	0.421
Si	0.81	3.10	0.963
$N\text{-}NO_3$	2.14	0.22	0.421
HCN	++	–	–

++: high content; – : absent; n.s.: non-significant

The differences in plant density, row spacing and maize:sorghum ratio in the various cultivation treatments caused no significant modification in the chemical composition of the plant material intended for ensiling (Table 4). Only in treatment D (with a sorghum-maize row ratio of 2:1) was there a higher concentration of structural carbohydrates and lignins.

Table 4
Chemical composition of the raw material for ensiling obtained from different cultivation technologies of sorghum and maize (g kg^{-1} DM)

Component	Treatment C	Treatment D	Treatment E	$LSD_{0.05}$
Crude protein	92.51	93.72	91.91	n.s.
Sugars	26.53	21.87	32.54	2.716
Cellulose	289.15	277.38	263.94	n.s.
Hemicelluloses	250.82	306.73	232.08	17.396
Lignins	12.13	35.53	28.68	4.818
Acid detergent fibre	301.24	312.81	292.5	13.361
Neutral detergent fibre	552.03	619.53	524.5	3.399
Crude ash	45.45	40.02	57.04	1.166
K	11.36	10.04	15.35	n.s.
Ca	6.88	6.01	5.876	0.438
Mg	1.94	1.73	2.644	n.s.
P	2.02	1.93	2.482	0.322
Na	0.73	0.13	0.684	0.261
Si	0.90	0.59	0.752	n.s.
$N\text{-}NO_3$	0.42	0.37	0.42	0.284
HCN	+	++	+	–

++: high content; + : low content; – : absent; n.s.: non-significant

Silage chemical composition

The silages obtained from pure stands of maize and sorghum and from experimental mixtures of these plants were of good quality. They were characterised by low pH and only traces of ammonium, while lactic acid was the dominant organic acid, with a content ranging from 31.25–51.58 g kg^{-1} DM (Tables 5 and 6). Butyric acid was found only in three silages in amounts which did not exceed 0.68 g kg^{-1} DM. Higher quantities of acetic acid were found in the silage from treatments A and E. As regards the basic silage composition, maize silage was found to have higher quantities of starch, which decreased when sorghum was added to the silage, with a concomitant increase in the content of reducing sugars. The concentration of protein tended to vary, but never exceeded a value of 113 g kg^{-1} DM. The contents of crude fibre, ADF and NDF in the experimental silages were similar to those found in silages from other crop plants, but the values of these constituents tended to increase in silage containing sorghum.

After incubation in the Rusitec apparatus all the rumen juice indices were comparable for all the silages and similar to the values obtained for other forages and when the rumen functioned normally (Table 7). The pH values did not exceed values of 6.96–7.34. Total LKP varied considerably, with the highest value (71.71 mmol/l) in treatment E and the lowest (44.32 mmol/l) in the 1:2 maize-sorghum treatment. Smaller differences were observed within the volatile fatty acid profile, although there was a statistically significant increase in the amount of propionic, isobutyric and butyric acids in treatment E. The quantities of bacteria and protozoa give some indication of the quality of rumen activity. These values ranged from 3.26–4.51 $\times$ 10^8 ml^{-1} for bacteria and 8.59–18.71 $\times$ 10^2 ml^{-1} for protozoa. It should be noted that silage containing sorghum exhibited higher values of protozoa.

Table 5

Chemical characteristics of silages from sorghum (treatment A) and maize (treatment B)

Item	Treatment A	Treatment B
pH	3.95	4.01
Dry matter (DM) (%)	20.16	29.47
Organic matter (%)	19.08	22.12
Ammonia (%)	0.03	0.04
Acetic acid (g kg^{-1} DM)	35.71	16.97
Propionic acid (g kg^{-1} DM)	2.98	3.39
Lactic acid (g kg^{-1} DM)	31.25	51.58
Butyric acid (g kg^{-1} DM)	0.51	0.68
Starch (g kg^{-1} DM)	251.98	346.11
Reducing sugars (g kg^{-1} DM)	77.38	36.65
Crude protein (g kg^{-1} DM)	87.32	96.03
Crude fibre (g kg^{-1} DM)	334.82	212.08
Crude ash (g kg^{-1} DM)	53.57	46.49
Acid detergent fibre (g kg^{-1} DM)	350.69	223.96
Neutral detergent fibre (g kg^{-1} DM)	571.92	430.27

Table 6
Chemical characteristics of silages from treatments with sorghum and maize

Item	Treatment C	Treatment D	Treatment E
pH	4.0	4.01	3.96
Dry matter (DM) (%)	24.46	27.2	25.19
Organic matter (%)	23.31	25.98	23.79
Ammonia (%)	0.04	0.03	0.08
Acetic acid (g kg^{-1} DM)	20.44	20.59	34.54
Propionic acid (g kg^{-1} DM)	3.68	2.21	6.75
Lactic acid (g kg^{-1} DM)	40.88	33.09	35.33
Butyric acid (g kg^{-1} DM)	x	x	0.4
Starch (g kg^{-1} DM)	327.88	297.74	251.98
Reducing sugars (g kg^{-1} DM)	59.69	73.44	77.38
Crude protein (g kg^{-1} DM)	86.67	98.9	113.14
Crude fibre (g kg^{-1} DM)	281.28	235.29	245.34
Crude ash (g kg^{-1} DM)	47.02	44.85	55.58
Acid detergent fibre (g kg^{-1} DM)	304.17	254.02	260.02
Neutral detergent fibre (g kg^{-1} DM)	518.4	475.37	503.37

x: not detected

Table 7
Indices of the rumen fluid following 10-day incubation

Item	Treatment B		Treatment C		Treatment D		Treatment E	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Rumen fluid								
pH	7.25^{a}	0.03	7.21^{a}	0.08	7.34^{a}	0.04	6.96^{b}	0.15
Ammonia (mmol/l)	0.80^{b}		0.55^{b}		1.22^{ab}		3.18^{a}	
Volatile fatty acids (VFA)								
VFA sum (mmol/l)	54.16^{b}	1.23	64.42^{ab}	2,45	44.32^{b}	0.52	71.71^{a}	0.62
Acetic (%)	51.3^{ab}	0.18	52.83^{ab}	1.36	48.2^{b}	1.70	53.71^{a}	2.11
Propionic (%)	18.1^{b}	0.07	19.31^{b}	0.98	20.74^{b}	1.03	28.81^{a}	0.98
Isobutyric (%)	1.18^{b}	0.88	2.66^{b}	0.11	2.69^{b}	0.30	4.64^{a}	0.75
Butyric (%)	8.85^{b}	0.73	10.65^{b}	0.46	11.1^{b}	0.91	17.99^{a}	0.44
Iso-valeric (%)	1.25	0.13	1.68	0.13	1.7	0.11	2.64	0.36
Valeric (%)	1.5	0.01	1.18	0.08	1.01	0.01	2.08	0.38
A/P	2.84^{a}	0.00	2.67^{a}	0.10	2.36^{ab}	0.06	1.85^{b}	0.01
Microorganisms in the rumen juice								
Bacteria (10^{8} ml^{-1})	4.12^{ab}	0.96	3.26^{a}	1.10	4.51^{a}	0.51	4.51^{a}	0.99
Protozoa (10^{2} ml^{-1})	8.59^{b}	5.63	14.13	5.13	18.71^{a}	3.92	16.16^{a}	3.13
Holotricha	0.19	0.43	0.38	0.52	0.38	0.52	0.12	0.16
Entodiniomorpha	8.40^{b}	5.92	13.75^{ab}	4.61	18.33^{a}	3.40	16.04^{a}	2.97

a,b: values designated with identical letters do not differ significantly $P<0.05$ level; A/P: proportion of acetic (A) to propionic (P) acids

The digestibility of dry matter and crude protein in the experimental silage was similar and ranged from 52.56–56.12% for dry matter and from 59.93–64.16% for crude protein. The digestibility values for ADF or even NDF crude fibre were relatively low and did not exceed 28% (Table 8).

Table 8
Digestibility of silages from maize and sorghum following 10-day incubation

Component	Treatment B		Treatment C		Treatment D		Treatment E	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Dry mass (%)	53.29^{ab}	3.88	54.15^{ab}	5.40	52.56^{b}	7.04	56.12^{a}	3.18
Crude protein (%)	59.93^{b}	1.12	61.29^{ab}	1.02	64.16^{a}	3.23	64.17^{a}	0.87
Fibre (%)	27.87^{a}	1.01	28.15^{a}	0.98	12.45^{b}	1.56	24.27^{ab}	0.954
Acid detergent fibre (%)	19.16^{ab}	0.75	22.29^{a}	1.78	9.31^{c}	0.78	17.64^{b}	0.85
Neutral detergent fibre (%)	20.35^{ab}	2.78	23.19^{a}	1.03	15.59^{b}	1.56	24.51^{a}	2.10

a,b: values designated with identical letters do not differ significantly at the $P<0.05$ level

Discussion

The appearance of sugar sorghum on the Polish fodder market raises fundamental questions about the nutritional and agronomic value of this plant species, which is primarily affected by the biological and chemical properties of the species and its cultivars (Madhavan and Shanmugasundavam, 1990; Faheed et al., 2005; Sheoran and Rana, 2005; Uptmoor et al., 2006). The analysis of these properties was the principal aim of the present investigations.

The analysis of plant chemical composition showed sorghum to be an interesting forage grass, with higher sugar and protein contents than maize. There were greater differences in these constituents in the leaf blades than in the stems. In addition the mineral composition of sorghum forage is close to optimal. The results obtained in this study are in good agreement with earlier studies (Kozłowski et al., 2006) and with the findings of Daczewska and Ostrowski (1986).

The chemical composition of cut aboveground parts intended for ensiling can be considered as favourable for the production of good quality silage. The different cultivation technologies did not change the chemical composition of sorghum forage, but they did influence the yield.

The yield of aboveground parts is the chief asset of sorghum as a fodder plant. In the light of the results (Table 9), sorghum can be considered as a complementary plant for maize. The results also confirm the outcome of breeding experiments carried out in Poland by the Main Research Centre of Crop Plants (Magda, 1980; Daczewska and Ostrowski, 1986). However, due to weather conditions (satisfactory amount of precipitation during the vegetation period and moderate air temperatures), sorghum was unable to exhibit its yield potential, as the species does best at high air temperatures, as reported by Magda (1980) and Śliwiński and Brzóska (2006).

Table 9
Aboveground yield in the different cultivation treatments

Treatment	Yield t ha^{-1} DM
A	12.427
B	18.855
C	13.712
D	12.070
E	12.684
$LSD_{0.05}$	0.7368

The high yield potential of sorghum is also reflected in its vitality, expressed in the chlorophyll content (Zielewicz and Kozłowski, 2007). One of the great advantages of maize as a fodder grass is the high proportion of ears in the stem structure, as reported by Cygert et al. (2006) and Kozłowski et al. (2006). It is the maize kernels that principally determine the nutritive value of the forage crop. The proportion of ears in the maize plant structure reached 43%, whereas the proportion of inflorescences in the sorghum plant structure was much smaller – approximately 14%. It is thus clear that the nutritive value of sorghum forage is influenced, primarily, by the chemical composition of the stems and leaf blades.

The analysis of the chemical composition of the silages revealed that the various mixtures of maize and sorghum used as raw material did not have a negative effect on the ensiling properties of the forage and that the silage quality was similar to that of pure maize silage (Stryszewska and Pyś, 2006). Nevertheless, there may be some differences in the content of certain nutrients, such as starch, crude fibre or NDF (Orosz et al., 2005; Śliwiński and Brzóska, 2006). Experiments carried out *in vitro*, or using the *in sacco* method employed for roughage fed to ruminants, provide a wide range of new information essential for the proper assessment of these feeds and their rational application (Filya, 2003).

In the present studies, the pH of the rumen juice obtained from mixed maize/sorghum silage was similar to that recorded in the Rusitec apparatus for silage made from grass/alfalfa mixtures (7.01–7.07); the concentrations of individual volatile fatty acids were also similar (Potkański et al., 2005). The bacterial and protozoa counts recorded in the present experiments were similar to those obtained with a different *in vitro* technique (batch culture) for grass/alfalfa silages (Cieślak et al., 2005). The bacterial counts recorded in the present experiments ranged from 3.26–4.51 $\times$ 10^8 ml^{-1}, compared with 2.44–3.36 $\times$ 10^8 ml^{-1} for grass/alfalfa silage (also using the Rusitec apparatus), and 12.33–15.67 $\times$ 10^8 ml^{-1} for the batch culture method. In the case of protozoa, the counts obtained in the present investigations ranged from 0.86–1.87 $\times$ 10^3 ml^{-1}, and in experiments on grass/alfalfa silages in the Rusitec apparatus from 0.69–2.17 $\times$ 10^3 ml^{-1}. These results confirm that the microbial activity in the rumen when feeding silage prepared from maize and sorghum was similar to that reported under *in vitro* conditions for other feeds, e.g. silage prepared from grasses with the addition of legumes. In order to obtain a better characterisation of the protozoa, a separate count was made of protozoa from the *Entodiniomorpha* genus and the *Holotricha* subclass. It was found that the most numerous protozoa in the rumen juice were *Entodiniomorpha.*

The values obtained for silage dry matter and crude protein digestibility did not exceed those reported in earlier investigations. The highest protein digestibility was obtained in treatments D (sorghum to maize ratio 2:1) and E (sorghum sown at a distance of 12 cm from the maize rows), with values of

64.16% and 64.17%, respectively, while the lowest protein digestibility was found in the silage produced from maize alone (treatment B – 59.93%). All the digestibility values for crude fibre, ADF and NDF were very low. The lowest values, which differed significantly from the results of other treatments, were recorded in treatment D.

The objective of these studies was to investigate the biological and chemical properties of sugar sorghum, which would justify and confirm the possibility of cultivating this plant under the climatic and soil conditions of Wielkopolska and similar regions. The feasibility of sorghum cultivation was confirmed by the studies of Daczewska and Ostrowski (1986) and Magda (1980), which included the Hungarian cultivars Hybar MV 301 and Szarvasi 480, as well as by our earlier experiments (Kozłowski et al., 2006).

Conclusions

– *Sorghum saccharatum* is a valuable fodder grass characterised by favourable biological and chemical properties, the most important traits being high crude protein content and moderate contents of structural carbohydrates and lignins. However, the tendency of the plant to accumulate nitrate nitrogen and cyanogenic glucosides may give rise to reservations. Another advantage of sorghum is its considerable yield potential.

– Sugar sorghum is a fodder grass complementary to maize. The different stem morphological structures of sorghum and maize may give rise to different quality assessments of the silage obtained from these grasses.

– Sorghum cultivation provides nutritionally valuable forage, which constitutes important silage material. The good chemical composition and digestibility of sorghum allow high quality silage to be prepared, irrespective of the technology employed.

– The different sowing combinations had no influence on the chemical composition of the forage and silage, so the production technology is of secondary importance, and the decisive factor is the yield of aboveground parts.

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EFFECT OF GAMMA RADIATION ON ANTIOXIDANT ENZYMES AND G_6PDH ACTIVITIES IN *Vicia faba* PLANTS

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The effect of gamma irradiation on *Vicia faba* L. plants was investigated by exposing dry seeds to doses ranging from 0 to 100 Gray (Gy) and studying the activities and isozyme patterns of the key enzymes involved in oxidative stress defence, such as superoxide dismutases (SOD, EC 1.15.1.1), catalases (CAT, EC 1.11.1.6), peroxidases (POX, EC 1.11.1.7), ascorbate peroxidases (APOX, EC 1.11.1.11), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and glutathione reductase (GR, EC 1.6.4.2), as well as the activity of an enzyme involved in a specific intermediary metabolic pathway, glucose-6-phosphate dehydrogenase (G_6PDH, EC 1.1.1.49). The H_2O_2 contents of faba bean leaves were also measured. None of the γ-irradiation doses used (0–100 Gy) had any effect on the activity of MDHAR, but they increased the enzyme activities of GR, APOX, SOD and G_6PDH. Gamma rays at 20 Gy decreased the H_2O_2 content, but the 100 Gy dose significantly increased the H_2O_2 content compared with the non-irradiated plants. The results implied that the isozymes of SOD, CAT and POX present in faba bean cells growing in the presence of 0–15 Gy γ-irradiation are required to remove the reactive oxygen species (ROS) produced during normal, physiological processes. When the dose of γ-irradiation is ≥20 Gy, the level of ROS (produced indirectly by γ-irradiation) becomes too high to be dealt with by the existing antioxidant isozymes. The present research shows for the first time that the switch between the physiological oxidative response and a stress-related one occurs within a very narrow range of stress factor intensities, i.e. γ-irradiation doses. In the present study, this change took place between 15 and 20 Gy. Further investigations, using molecular biology techniques will be needed to determine the mechanisms involved in enzyme induction under ionizing conditions in order to evaluate changes in the gametic genomes at two possible levels: (i) the structural level, for studying mutations occurring in the DNA, and (ii) the functional level, by studying differential genetic expression between irradiated and non-irradiated plants.

Key words: photosynthesis, gamma irradiation, antioxidant enzyme, *Vicia faba* L.

Introduction

Vicia faba L. is considered to be one of the world's most important legume crops. In Egypt, this plant occupies an area of 150,000 ha and represents a major food crop. It is also used for animal feed and industrial purposes. It is well established that, whatever its nature, stress causes the production of a large amount of highly reactive oxygen species (ROS) (Moussa, 2006). In aerobic organisms, moreover, an enhanced production of O_2^- (superoxide anion) and H_2O_2 takes place. These latter species are relatively less harmful, but they can enter the 'Fenton' reaction catalysed by a metal ion (Fe^{2+}) and generate the highly aggressive OH$^\bullet$ radical (Wardman and Candeias, 1996). A large amount of biological damage is caused by this radical, which reacts with almost all structural and functional organic molecules, including proteins, chlorophyll, lipids and nucleic acids (Becana et al., 1998). OH$^\bullet$ can cause the peroxidation of unsaturated membrane fatty acids, forming peroxyl (ROO$^\bullet$) and alkoxyl (RO$^\bullet$) radicals (Salter and Hewitt, 1992), resulting in a loss of cellular compartmentation and thus causing metabolic disturbance. Its oxidative attack on proteins (Wolff and Dean, 1986) can greatly alter their properties and functions. H_2O_2 is known to be a toxic compound that is produced as a result of the scavenging of superoxide radicals. Higher concentrations are injurious to plants as a result of lipid peroxidation and membrane injury (Nayar and Kaushal, 2002). Living organisms, particularly photosynthetic organisms, are continuously exposed to ROS, but their exposure is significantly enhanced under oxidative conditions. For this reason, they have evolved efficient enzymatic and non-enzymatic detoxifying systems to overcome damage due to ROS (Larson, 1988). Knowing that water radiolysis, the predominant effect of ionizing radiation in organisms, induces ROS formation (De Vita et al., 1993), one can assume that the plant, bacterial and animal enzymes that are involved in cell protection against oxidative stress will display similar responses under ionizing radiation stress as under other stress factors. It is hypothesized that the modulation of the activities of these enzymes in early growth stages may be important in imparting resistance to a plant against environmental stresses (Becana et al., 1998). With the rising problem of environmental radioactive pollution, generating relatively low radiation doses in contaminated areas where agriculture is the major activity, it is necessary to collect reliable data on the effects of such radiation on biological organisms (Zaka et al., 2002). There is little information concerning the effects of ionizing radiation stress on the activity of the antioxidant enzymes in plants. Thus, in this study, an attempt was made to answer the following question: does external γ-irradiation significantly modify the activity of oxidative stress defence enzymes? In order to respond to this question, the activities of key enzymes involved in oxidative stress defence, such as SOD, CAT, POD, APOX, MDHAR and GR, were studied, together with the activity of an enzyme involved in a specific intermediary metabolic pathway, G_6PDH, which provides NADPH for the efficient functioning of the ascorbate–glutathione pathway and of other H_2O_2 scavenging systems such as flavonoids (Shimoi et al., 1996). This enzyme, not indispensable for survival under normal conditions, is essential in cell defence against oxidative stress (Pandolfi et al., 1995).

Materials and methods

Irradiation and cultivation

Uniformly sized faba bean seeds (*Vicia faba* L.) cv. Giza 2, purchased from the Crop Institute, Agriculture Research Center, Giza, Egypt, were surface sterilized in 0.1% (w/v) sodium dodecyl sulphate (SDS) solution and then thoroughly rinsed with sterile deionized water. Dry seeds were exposed to different doses of gamma irradiation (0.0, 2, 5, 10, 15, 20, 30, 50, 80 and 100 Gy), using a gamma source (^{60}Co), at the Middle Eastern Regional Radioisotope Center for the Arab Countries (Dokki, Cairo, Egypt) with a strength of 500 Ci and a dose rate of 0.54 Gy/min. The seeds were planted in black polyethylene pots (30 cm high × 40 cm diameter) filled with peat, sand and soil (1:1:1) and grown for three weeks at a day/night temperature of 24/18°C, with 70% relative humidity, 14 h light and a photon flux density of 400 μmol m^{-2} s^{-1}. Cultural practices, such as weed control and irrigation, were performed as needed. Five replicates of each treatment were prepared to give a total of 50 pots. Ten seedlings per dose (including the control) were grown. The results were compared with those obtained in a control set of non-irradiated seedlings submitted to the same experimental process from imbibition to cultivation.

Biochemical assays

Proteins were extracted by grinding 100 mg of frozen leaf material in a mortar in liquid nitrogen and homogenizing it in 1 mL of a buffer solution. The extraction buffer for leaf proteins, used for the determination of the activity of most enzymes, consisted of 0.1 M TRIS–HCl (pH 7.5), 0.23 M sucrose, 20% PVP (polyvinylpyrrolidone), 4 mM ß-mercaptoethanol, 1 mM EDTA, 10 mM KCl and 10 mM $MgCl_2$. Five mM ascorbic acid was added to the buffer just before use. To extract proteins for the determination of APOX and MDHAR, 5% PVP was used and the ß-mercaptoethanol was omitted. After homogenization and centrifugation (10,000 g at 4°C for 20 min), the supernatants were used for enzyme assays. APOX and GR assays were performed according to Vanaker et al. (1998), MDHAR according to Miyake and Asada (1992), SOD according to Dhindhsa et al. (1981) and G_6PDH according to Aoki et al. (1998). Samples of crude faba bean extracts were electrophoresed in 10% (SOD and POX) or 8% (CAT) (w/v) polyacrylamide slab gels at pH 8.9 under non-denaturing conditions (Davis, 1964). Isozymes of the antioxidant enzymes were visualized in gels by the methods of Beauchamp and Fridovich (1971) for SOD, Woodbury et al. (1971) for CAT, and Ros Barceló et al. (2002) for POX. The contents of hydrogen peroxide in faba bean leaves were measured according to Patterson et al. (1984). The statistical analyses were performed with the *STATISTICA®* software package (StatSoft, 1999).

Results

None of the γ-irradiation doses used (0–100 Gy) had any effect on the activity of MDHAR. Gamma rays at 20 Gy increased the activities of GR by 87.5%, SOD by 12.6%, APOX by 22.9% and G_6PDH by 38.9% and decreased the H_2O_2 content by 17.8% as compared with non-irradiated plants (Table 1). Meanwhile, high doses of gamma radiation (100 Gy) significantly increased the activities of GR by 275%, SOD by 32.9%, APOX by 153.4% G_6PDH by 238.9%, and the H_2O_2 content by 191.1% as compared with the control plants (Table 1). Total SOD activity was affected by different doses of γ-irradiation, and the isozyme pattern of SOD was significantly different at growth-inhibiting doses of γ-irradiation (≥20 Gy) (Fig. 1) compared with non-inhibiting ones. The most visible effect was the gradual disappearance of isozymes II, III, IV and V,

with increasing γ-irradiation doses with the concomitant appearance of isozymes VI, VII, VIII, IX and X. The activity of isozyme I was the same in control cells as it was in those treated with various doses of γ-irradiation. Total CAT activity was lower in faba bean cells treated with growth-inhibiting γ-irradiation doses (≥20 Gy) compared with control cells or those treated with various low doses of γ-irradiation (2–15 Gy) (Fig. 2). CAT is represented by one isozyme (I) in cells growing in the presence of 0–15 Gy and by another (II) within cells treated with higher doses of γ-irradiation (≥20 Gy). The activity of CAT I increased gradually in control cells and those grown at 2–15 Gy, whereas the activity of CAT II was highest at a dose of 20 Gy and decreased at higher doses of γ-irradiation (30–100 Gy). The POX activity and isozyme pattern were strongly affected by higher doses of γ-irradiation (≥20 Gy), at which new isozymes, designated as III, V and VI, appeared. The activities of other isoforms (I, II, IV) were enhanced compared with the control cells or those treated with lower doses of γ-irradiation (2–15 Gy) (Fig. 3). The results implied that the isozymes of SOD, CAT and POX present in faba bean cells growing in the presence of 0–15 Gy of γ-irradiation are required to remove the ROS produced during normal, physiological processes. When the dose of γ-irradiation is ≥20 Gy, the level of ROS (produced indirectly by γ-irradiation) becomes too high to be dealt with by the existing antioxidant isozymes. Thus, faba bean cells switch on additional antioxidant enzyme-dependent defence reactions, which involve novel isozymes. The present research shows for the first time that the switch between the physiological oxidative response and a stress-related one occurs over a very narrow range of stress factor intensities, i.e. γ-irradiation doses. In the present study, this change took place between 15 and 20 Gy.

Table 1

Changes in enzyme activities of GR (μmol $NADP^+$ min^{-1} mg^{-1} protein), SOD (unit mg^{-1} protein), APOX (μmol oxidized ascorbic acid min^{-1} mg^{-1} protein), MDHAR (μmol $NADP^+$ min^{-1} mg^{-1} protein), G_6PDH (μmol NADPH min^{-1} mg^{-1} protein) and H_2O_2 (μmol g^{-1} FW) in faba bean cells treated with different doses of γ-irradiation (0–100 Gy) for three weeks

γ-Irradiation doses (Gy)	Enzyme assay					H_2O_2
	GR	SOD	APOX	MDHAR	G_6PDH	
0.0	8	143	1.61	2.11	18	34
2	9	149	1.53	2.09	16	30
5	10	142	1.62	2.10	19	29
10	12	151	1.71	2.12	21	31
15	13	154	1.78	2.13	23	33
20	15	156	1.98	2.14	25	30
30	18	161	2.31	2.18	29	48
50	21	168	2.83	2.15	33	59
80	25	173	3.75	2.13	40	71
100	30	190	4.08	2.17	51	99
LSD 1%	2.2	4.1	0.9	0.8	2.9	3.1
LSD 5%	1.6	2.9	0.5	0.5	1.9	1.8

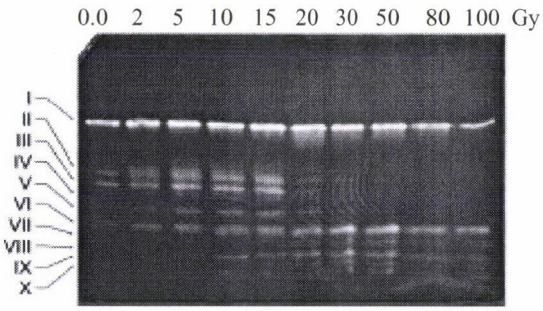

Fig. 1. Isoenzyme pattern of SOD in faba bean leaves treated for three weeks with different doses of γ-irradiation (0–100 Gy)

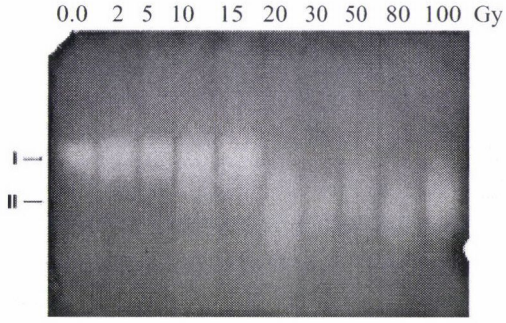

Fig. 2. Isoenzyme pattern of CAT in faba bean leaves treated for three weeks with different doses of γ-irradiation (0–100 Gy)

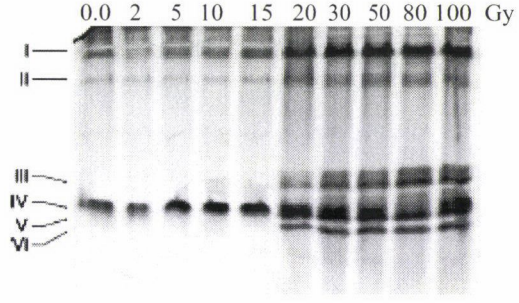

Fig. 3. Isoenzyme pattern of POX in faba bean leaves treated for three weeks with different doses of γ-irradiation (0–100 Gy)

Discussion

Plants are well known to possess enzymatic and non-enzymatic detoxifying systems continuously involved in cellular protection against ROS arising from both the environment and the cell metabolism. In the case of APOX stimulation, a similar response has been observed in wheat growing in a saline

environment (Meneguzzo et al., 1998) and in tobacco under UVB radiation (Willekens et al., 1994). According to Karpinski et al. (1997), the APOX activity induced in *Arabidopsis* subjected to oxidative stress conditions, such as high light intensity, takes place by the induction of *APOX1* and *APOX2* gene transcription. One can assume that this induction of the genetic expression of APOX can also take place in cells undergoing γ-radiation stress. These enzymes (APOX, MDHAR and GR) belong to the same metabolic pathway (Halliwell and Asada pathway); they may be diversely involved in protection against gamma radiation. These results agree with those of Gupta et al. (1993a, b) on tobacco, in which resistance to oxidative stress is due to the over-expression of Cu/ZnSOD and APOX activities, while MDHAR and GR activities are not affected. In *Arabidopsis thaliana* as well, Kubo et al. (1995) observed that a one-week exposure of the plants to O_3 or SO_2 had only a slight effect on the activity of the same enzymes. Considering their results, it can be assumed that in faba bean under ionizing conditions the recycling of the oxidized form of ascorbate to the reduced form would involve DHAR (dehydroascorbate reductase) rather than MDHAR. In this case, GR would also be involved in the recycling of the electron donor (GSH) later in the pathway. A slight increase in GR activity occurred, probably due to the enhancement of the transcription rate of the encoding genes (Foyer et al., 1991). The hypothesis of GR radio-induction in faba bean is supported by the fact that a similar response was also obtained in these plants for G_6PDH. Indeed, this enzyme plays a major role in the ascorbate–glutathione pathway, supplying GR and MDHAR with NADPH. G_6PDH activity is also necessary for the activity of the thioredoxin–NADPH-dependent system (Fridovich, 1983). Both are considered key systems ensuring the protection of the plant cell against oxidative damage. The stimulation of SOD activity is possibly due to a positive regulation of SOD genes or of one particular encoding allele, in response to γ-irradiation stress, as shown in different biological models (Inzé and Van Montagu, 1995). It is also important to emphasize that SOD induction seemed to depend on the gamma radiation dose, indicating that the acquisition of SOD gene regulation, as well as that observed for POX under the same conditions, is a function of the severity of the ionizing conditions. This over-expression could be compared with that observed in various plant or animal systems subjected to pre-irradiation, which led to an increase in free radical scavenging ability at higher irradiation doses. For example, this adaptation to environmental stress was experienced in rat hepatic cells (Yukawa et al., 1999), and in vascular endothelial bovine cells, where CAT and POX activities were stimulated after exposure to H_2O_2 pretreatment (Lu et al., 1993). In the same way, when CAT, a thermo-labile enzyme, was exposed to 14°C pretreatment, it resulted in the elevation of the H_2O_2 level and led, during maize germination, to a stimulation of *cat-3* expression at temperatures below 4°C (Prasad et al., 1994). The same treatment generated a similar response for POX and SOD in zucchini (Wang, 1995). This over-expression probably occurs due to adjustments in the

regulatory mechanism. In this case, it is not surprising to note a strong stimulation of the genes encoding for SOD. The scavenging of H_2O_2 is then performed by other enzymatic and/or non-enzymatic antioxidant systems. The induction of G_6PDH can be explained in a similar way to that of SOD, because this enzyme of the intermediary metabolism provides NADPH for the efficient functioning of the ascorbate–glutathione pathway and of other H_2O_2 scavenging systems, such as flavonoids (Shimoi et al., 1996). This enzyme, not indispensable for survival under normal conditions, is essential in cell defence against oxidative stress (Pandolfi et al., 1995). The induction of stress-related isozymes is probably related to the level of ROS, which causes oxidative damage to various cellular components, such as proteins, membrane lipids and nucleic acids (Halliwell and Gutterdge, 1989).

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RESIDUAL EFFECTS OF PHOSPHORUS AND SOYABEAN CROP ON MAIZE IN THE GUINEA SAVANNA OF WEST AFRICA

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Two seasons of cropping were carried out at three sites in the Guinea savanna to evaluate the residual effects of soyabean on maize. The experiment was laid out as a split-plot design in a randomized complete block with three replications. In the first season, four soyabean varieties with a fallow treatment (control) received phosphorus (P) applied as triple superphosphate (20% P) at the rates of 30 and 60 kg P ha^{-1}. Maize was grown in these plots in the second season without fertilizer application. At all sites, regardless of the previous crop, total soil N remained low (<1.5 g kg^{-1}). Available P was affected by the P rate in the previous year at all sites. From initial values ranging from 5.2–16.2 mg kg^{-1} in the first season, available P significantly ($p<0.05$) increased in the second season to 9.8–42.8 mg kg^{-1} when 30 or 60 kg P ha^{-1} was applied, compared to 7.7–18.6 mg kg^{-1} at no P application. Relative to no P application in the previous year, the application of 60 kg P ha^{-1} significantly increased total dry matter at 6 weeks after planting by 19%, total harvest dry matter by 28%, and grain yield by 37%.

Key words: residual effect, soyabean, phosphorus, maize, Guinea savanna, cropping system, rotation

Introduction

The Guinea savanna agro-ecological zone is a major area for the production of cereals and grain legumes in West Africa. The savannas are, however, characterised by low effective cation exchange capacity, and N and P deficiencies (Ssali et al., 1986; Ker, 1995). The main cropping systems of this ecological zone have been cereal-based with fallowing. In many instances, however, fallow periods have been greatly reduced. This has led to soil nutrient depletion, acidification, compaction, and the build-up of pest problems (Weber et al., 1996). Increasing pressure on land has resulted in the widespread practice of continuously cropping farm land without adequate nutrient input. However, apart from the problems of cost and unavailability, the use of inorganic N

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fertilizers to replenish soil N is fraught with the pollution of underground water, acidification and deleterious effects on soil fauna. The inclusion of legumes, on the other hand, offers a cleaner and more sustainable alternative for the improvement of cropping systems. When legumes are planted either alongside other crops in intercropping situations or in rotation cycles, they have the potential to contribute significantly to maintaining N levels, organic matter and physical soil properties for intensifying cereal-based cropping systems (Danso, 1992; Bohlool et al., 1992; COMBS, 1993). For instance, Ståhl et al. (2002) reported that N addition by *Sesbania sesban* resulted in significantly higher maize yields compared to continuous maize cropping. Generally, biological nitrogen fixation in legumes contributes almost 20% of the nitrogen (N) needed for world grain and oilseed production (Herridge and Rose, 2000). Low available soil P is, however, a limitation in the production of grain legumes (Leidi and Rodriguez-Navarro, 2000). Under low soil P status, P fertilizer application and its management are of importance in attaining high yields in legumes like soyabeans. Phosphorus is not only essential for plant growth, its availability has been noted to affect the functioning of the biological nitrogen fixation system in legumes (McLaughlin et al., 1990; Chien et al., 1993). Also, because maize is responsive to both direct and residual P, the application of P has to be done within the cropping system. This will ensure economy, cost effectiveness and the conservation of depletable resources (Goswami et al., 1990). Information is, however, scarce on the residual effects of P applied to soyabean crops on the subsequent maize crop in the Guinea savanna. Such information will lead to the development of specific P management practices for cereal-based cropping systems in this agro-ecology.

Materials and methods

This experiment was laid out as a split-plot design in a randomized complete block with three replications at three sites in the Guinea savanna. Four promiscuously nodulating soyabean varieties, namely TG×1670-1F and TG×923-2E (late duration), TG×536-02D (medium duration) and TG×1485-1D (early duration), were planted at all sites in the first season. Fallow control plots were also established for each replication in the first season. In the first season also, phosphorus was applied as triple superphosphate (20% P) at 30 and 60 kg ha^{-1} to the soyabean varieties and fallow plots. Control plots received no P treatment. In the second season, maize variety TZECOMP4C2 was sown in all previous soyabean and fallow plots. Moisture from rainfall was available all through the growing periods of soyabean and maize at all three sites. The amounts of rainfall received are presented in Table 1. At land preparation in the first season, 20 core samples were randomly collected to 15 cm depth using a 10 cm diameter soil auger. In the second season, before the planting of maize, soil samples were taken from each plot. The soil samples were air-dried, crushed and sieved through 2 mm and 0.05 mm meshes for the characterisation of soil physical and chemical properties. Soil N and P contents were determined based on the procedures outlined by Okalebo et al. (1993). Soil pH was measured with a pH meter using a soil to water ratio of 1:1. Chemical analyses for soil samples in the first season showed that total soil N was low (0.60–1.13 g kg^{-1}) across the sites, while pH ranged from strongly acid (4.9) at Gidan Waya to slightly acid (6.1) at Mokwa and Fashola. Phosphorus was of medium availability (16.2 mg kg^{-1}) at Mokwa and very low ($\leq$ 6.2 mg kg^{-1}) at Fashola and Gidan Waya.

Table 1
Total rainfall at the experimental sites during the cropping seasons

Month	Rainfall (mm)					
	Mokwa		Fashola		Gidan Waya	
	1st season	2nd season	1st season	2nd season	1st season	2nd season
May	186	235	154	139	48	na
June	230	254	286	225	282	199
July	110	142	124	79	420	316
August	266	103	175	65	367	361
September	148	11	237	193	156	128
October	87	Hv	99	Hv	100	Hv
Total rainfall	1027	745	1075	701	1373	1004

na = Rainfall data not available; Hv = maize crop alreadyharvested

Soyabeans were harvested after the leaves and petioles had fallen. At this time the pods had turned brown. Standing plants were cut at the base and the pods removed and threshed. Stover and pod walls were not returned to the field. Since this report is focused on residual effects, the data discussed were derived mainly during the second season. At 6 weeks after planting (WAP) in the second season, five maize plants were destructively harvested from within the five central rows of each plot. No border plants were sampled. Each plant was cut above ground and separated into leaf and stem. These samples were first air-dried and then oven-dried for 48 hours at 65°C to determine the dry matter contents. At final harvest in maize, all plants in the three central rows of each plot (the row length harvested was 3 m) were cut above ground. The harvest area was 1 m from each end of the rows. The maize ears were separated from the stover and the total field weight determined for each plot. Dry matter was determined for each plot by drying ten representative samples of maize ears and five of stover at 65°C for 48 hours. After shelling, the moisture content of the grain samples was determined using a Dickey-John moisture meter (Dickey-John Co., Auburn, IL, USA). Grain yields were corrected to 12% moisture content. Analysis of variance (ANOVA) was carried out using the SAS package (SAS, 2002). Duncan's Multiple Range Test (DMRT) was used to compare the means of the main effects and their interactions.

Results and discussion

At the beginning of the second season, that is, after cropping soyabeans or maintaining fallow plots in the first season, the total soil N content was not significantly different between the previous crop plots at Mokwa and Fashola. At all sites, however, including Gidan Waya (where there was a highly significant difference between the plots), total soil N remained low (0.36–1.34 g kg^{-1}) regardless of the previous crop (Table 2). Enwezor et al. (1989) reported that soils with a total N content of less than 1.5 g kg^{-1} have a low N rating. Although soyabeans are able to fix atmospheric N, it was reported that 14–36% of total plant N was derived from the soil by soyabeans at these experimental sites (Ogoke et al., 2003). In addition, soyabean residues were not returned after threshing. Nutrient inputs from soyabeans were, therefore, limited to the roots and litter. Total soil N in previous soyabean plots (where all aboveground plant biomass was exported) was comparable to total soil N in previous fallow plots,

where no nutrient export occurred through biomass export. This implies a possible build-up of total soil N with the return of soyabean residues after threshing and/or intensified soyabean cultivation. Available P contents were not significantly different between previous crop plots at Mokwa and Fashola. At Gidan Waya, where ANOVA showed a significant difference between previous crop plots, the available soil P in the previous TG×1670-1F plots (10.3 mg kg^{-1}) was significantly lower compared to other plots. This variety is late maturing and has been reported to have the highest P recovery among the varieties tested (Ogoke et al., 2006). In all previous crop plots, available P ranged from 20.9 mg kg^{-1} (medium) for TG×1485-1D to 39.1 mg kg^{-1} (high) for TG×1670-1F at Mokwa. At Fashola, available P remained low with little increase. For all plots of previous crops at Gidan Waya, available P increased from the initial level of 6.2 to 10.3 mg kg^{-1} for previous TG×1670-1F crop and 20.3 mg kg^{-1} for previous TG×1485-1D crop. Soil pH ranged from strongly to moderately acid at Gidan Waya (Table 2). At Fashola, soil pH remained slightly acid. Soil pH at Mokwa decreased slightly but remained moderately acid for all previous crop plots.

Total soil N content, pH and available P are shown in Table 3 as affected by P application in the previous year. At all sites total soil N remained low in the year following P application. It was expected that adequate soil P would enhance atmospheric N fixation in soyabeans and lead to soil N build-up through the decomposition of soyabean plant parts left in the fields. There was, however, no significant effect of the previous P rate on total soil N at Mokwa or Fashola. At Mokwa, the initial available soil P level of 16.2 mg kg^{-1} was in the range of medium availability, which is well above the critical P requirement, determined to be 10.5 mg kg^{-1} for soyabean (Aune and Lal, 1995). Because initial soil N was already sufficient at this site, the application of P could not significantly increase soil N compared to when no P was applied. Initial soil P was so low at Fashola that even P application at 60 kg ha^{-1} could not raise the soil P above the range of low availability, although this was higher than the critical level for soyabeans. Although P application in the previous year had a significant effect on total soil N at Gidan Waya, values remained low, as at other sites. At this site, total soil N was significantly higher when 60 kg P ha^{-1} was applied in the previous year, indicating that more N might have been derived from the soil at lower P rates. Generally, P application raised available P in the year after application. Available P determined by Bray II was significantly affected by the P rate in the previous year at all sites. It was lowest when no P was applied in the previous year and highest at a previous P rate of 60 kg P ha^{-1}. While soil pH fell from slightly acid to moderately acid at Mokwa for all previous P levels, it remained slightly acid at Fashola and very strongly acid at Gidan Waya (Table 3). Statistical analysis, however, showed that soil pH was not significantly affected by the previous P rate at Mokwa and Fashola. At Gidan Waya, where it was significant, the difference of 0.1 was small, while soil pH remained very strongly acid.

Table 2
Effects of previous crops of soyabean and fallow on soil pH, total N and available P

Soil property/site	1996 value	Previous crop					Significance level
		TG×1485-1D	TG×536-02D	TG×923-2E	TG×1670-1F	Fallow	
Total N (g kg^{-1})							
Mokwa	0.60	0.39	0.56	0.57	0.52	0.59	ns
Fashola	0.69	0.67	0.61	0.70	0.73	0.65	ns
Gidan Waya	1.13	1.17a	1.34a	1.22a	0.88b	1.00b	**
Available P (mg kg^{-1})							
Mokwa	16.2	20.9	31.2	30.4	39.1	36.2	ns
Fashola	5.2	11.1	7.8	10.8	9.8	10.0	ns
Gidan Waya	6.2	20.3a	17.3a	18.3a	10.3b	15.67a	**
pH (H_2O)							
Mokwa	6.1	5.9	5.7	5.7	5.8	6.0	ns
Fashola	6.1	6.2	6.2	6.2	6.1	6.2	ns
Gidan Waya	4.9	4.7b	5.2a	4.8b	5.2a	5.1a	**

ns = Not significant, ** = Significant at 1%; For each parameter at Gidan Waya, means for previous crop followed by the same letter are not significantly different

Table 3
Effect of phosphorus fertilizer rate in the previous year on soil pH, total N and available P

Properties/site	Pre-cropping value	Previous year P application (kg P ha^{-1})			Significance level
		0	30	60	
Total N (g kg^{-1})					
Mokwa	0.63	0.50	0.51	0.51	ns
Fashola	0.69	0.68	0.69	0.69	ns
Gidan Waya	1.13	0.92 b	1.03 b	1.19 a	**
Available P (g kg^{-1})					
Mokwa	16.2	18.6 b	33.7 a	42.8 a	**
Fashola	5.2	7.9 b	9.8 ab	13.6 a	*
Gidan Waya	6.7	7.7 c	15.9 b	26.4 a	**
pH (H_2O)					
Mokwa	6.1	5.8	5.8	5.8	ns
Fashola	6.1	6.2	6.3	6.2	ns
Gidan Waya	4.9	5.0 a	5.0 a	4.9 b	*

ns = Not significant, ** = Significant at 1%, * = Significant at 5%; Means of previous year P rates for each site followed by the same letter are not significantly different at the given level of significance.

The results from this study show that the total maize dry matter, at 6 weeks after planting and at grain harvest, and the grain yield were not significantly affected by the previous crop. Considering that no plant biomass was removed from the fallow plots, fallow vegetation was able to recycle nutrients from different depths in the fallow plots without the export of nutrients from the plots. In the soyabean plots, however, the standing plant biomass was removed at grain harvest and harvest residues were not returned after threshing. Consequently, not all the nutrients accumulated in the aboveground biomass were returned, implying some level of nutrient depletion. In spite of this,

previous soyabean crops were comparable to the fallow plot in total maize dry matter yield. Some residual contributions therefore arose from the soyabean crops. This has previously been quantified and reported as nitrogen fertilizer replacement values (Ogoke et al., 2001).

The P rate in the previous year and the site × previous year P rate interaction had a significant ($p<0.05$) effect on total dry matter at 6 WAP and a highly significant ($p<0.01$) effect on maize total harvest dry matter and grain yield. The site effect on harvest dry matter and grain yield was also highly significant (Table 4). The results showed an increasing residual P effect with increasing P rates applied the previous year for the total dry matter, harvest dry matter and grain yield. While Singh and Singh (1986) and DeMooy et al. (1973) observed that maize responded to both direct P application and its residual effect, Jat and Ahlawat (2006) reported that P applied to chickpea influenced the nutrient uptake in subsequent maize. Relative to no P application in the previous year, the application of 60 kg P ha^{-1} significantly increased total dry matter at 6 WAP by 19%, harvest dry matter by 28% and yield by 37%. The effects of 30 kg P ha^{-1} applied in the previous year on these parameters (except for yield) were not significantly different from when 60 kg P ha^{-1} was applied. The significant site × P rate interaction revealed by the analysis of variance procedures on all the parameters presented here may be attributable to the effects at Gidan Waya, where the soil reaction was strongly acid. At this site the increase in available P was higher compared to other sites. From a low level of 6.2 mg kg^{-1}, available P increased in the year following P application, by 156% at 30 kg P ha^{-1} and by 326% at 60 kg P ha^{-1}. Phosphorus application at higher rates may have resulted in more P being made available after fixation sites were occupied. The non-significant response of grain and stover yields to residual P, observed at Mokwa and Fashola despite the increase in available P, could be because N was the limiting nutrient and atmospheric N fixation did not lead to a substantial increase in the N available to the maize crop. At Mokwa, in the year following P application the available soil P contents for all plots given P fertilizer in the previous year (21–39 mg kg^{-1}) were higher than the critical P range (10–16 mg kg^{-1}) reported for maize (Sobulo and Osiname, 1981; Adeoye and Agboola, 1985). At Fashola, although available P increased in the year following P application, it was lower than the critical value for maize at all previous year P rates.

Conclusions

At low available soil P, applications of 30 kg P ha^{-1} or more to soyabean resulted in the raising of the available P level. In this study available P was increased to levels that should satisfy the P requirements of subsequent crops, in which case the further application of P may become detrimental, at least in the first year following application. One season of soyabean cropping was, however, not enough to raise soil N beyond the range of low availability. In addition, the

full benefits of the N contribution by soyabean may be obtained when all aboveground residues are returned.

Table 4

Effect of site and previous year P rate on maize dry matter and yield

P rate (kg ha^{-1})	Site			Mean of P
	Mokwa	Fashola	Gidan Waya	
		Total dry matter 6 WAP		
0	8.55ns	11.30ns	5.91b	8.59b
30	9.11ns	11.34ns	7.48b	9.31ab
60	9.72ns	10.84ns	9.96a	10.18a
Mean of site	9.13ns	11.16ns	7.78ns	
		Harvest dry matter		
0	3.01ns	1.48ns	1.75c	2.08b
30	3.14ns	1.54ns	2.35b	2.34ab
60	3.07ns	1.57ns	3.35a	2.66a
Mean of site	3.07a	1.53c	2.49b	
		Grain yield		
0	1.44ns	0.43ns	0.79b	0.89b
30	1.42ns	0.36ns	1.09b	0.96b
60	1.44ns	0.43ns	1.78a	1.22a
Mean of site	1.43a	0.41c	1.22b	

ns = not significant, WAP = weeks after planting; For each parameter, means of previous year P rates for each site followed by the same letter(s) are not significantly different at $p<0.05$ for total dry matter at 6 WAP, and at $p<0.01$ for harvest dry matter and grain yield

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Book review

M. BIRKÁS (Ed.): Environmentally-sound adaptable tillage. 2008. Akadémiai Kiadó, Budapest, Hungary. 354 pp. ISBN 978 963 05 8631 3

Regulated plant production requires soil tillage of the cultivated land. The form of production and the structure of cultivation have changed from time to time throughout history. The diagnosis of the effect of tillage operations on cultivated soils in the interests of sustainable crop production is now of vital importance. The team of contributors led by Professor Márta Birkás of Szent István University undertook the challenging task of providing the reader with a comprehensive picture of the results of research on environmentally sound adaptable tillage. Professor Birkás and three co-authors – András Szemők, Gábor Antos and Miklós Neményi – have written 9 chapters covering wide aspects of soil tillage. The reader is supplied with a wide range of knowledge on the assessment of soil conditions, the aims and energy requirements of soil tillage, the effects of site factors, the influence of biological factors, adaptable and environmentally-focused tillage, the practical applicability of environment conservation and energy-saving tillage, and environmentally sound land use. The last chapter gathers the lessons drawn from the history of tillage. The book contains information on conventional and new tillage techniques aimed at the reduction of environmental damage such as compaction, moisture loss, and the deterioration of soil structure and biological activity. Great emphasis is placed on presenting the main advantages and considerations of intensive, low intensity, integrated and ecological land use patterns. Informative and convincing experimental data are provided on each aspect of conventional and adaptable, environmentally-focused tillage.

In each chapter short informative tables as well as illustrative drawings and pictures help the reader to understand the advantages and disadvantages of tillage. The high value of the book is given not only by the synthesising, demonstrative grouping of the experimental data and findings, but also by the *References* section, which provides a complete list of the literature (170 publications). It is almost impossible in a brief review to introduce the multiplicity of tillage aspects covered by the book, all of which are directly or indirectly connected with tillage practices. The well-designed, unified, logical structure followed in each chapter is very impressive. It focuses on the good and unfavourable effects of tillage operations, besides giving the most relevant information on the technical aspects of tillage. The tillage effects considered range from the well-known phenomenon of compaction through changes in soil moisture status to weed infestation and earth worm number count. Putting the chapter on the history of tillage at the end of the book emphasises the lessons drawn, which are utilised in modern adaptable tillage practices. The references will be of assistance to readers who wish to know more about the different tillage practices or their consequences. Among the literature the reader will find publications from neighbouring countries, presenting expert opinion from a wider but similar region. Due to the multiplicity of fields it covers, this book is a must for both public libraries and agricultural research institutions. It will be of interest to specialists wishing to obtain information on the results achieved in related fields of soil cultivation, to university students, and to those working in plant production firms, but will also provide valuable information for farmers and for those interested in the topic of modern adaptable soil cultivation.

K. RAJKAI

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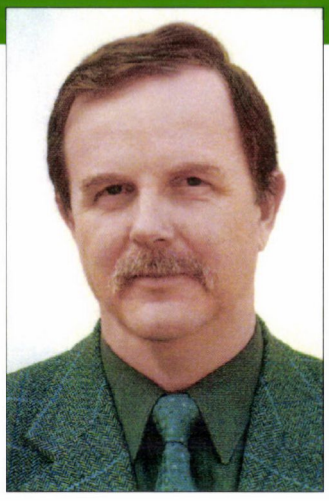

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STUDIES ON THE EFFECT OF N FERTILISATION ON THE GROWTH OF MAIZE (*Zea mays* L.) HYBRIDS I. DYNAMICS OF DRY MATTER ACCUMULATION IN WHOLE PLANTS AND PLANT ORGANS

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Investigations on the process of dry matter accumulation over time could contribute to improvements in the N fertiliser utilisation of maize hybrids. In 2001 and 2002 the effect of four N fertiliser rates (0, 80, 160, 240 kg ha^{-1}) on the growth and productivity of three maize hybrids with different vegetation periods [Mv TC 272 (FAO 280), Mv 355 SC (FAO 390) and Maraton SC (FAO 450)] was studied in an almost 50-year-old long-term experiment involving continuous maize, as a stress environment. The experiment was set up in Martonvásár on chernozem soil with forest residues in a split-plot design with four replications, with the N treatments in the main plots and the maize hybrids in the subplots. Plant samples for yield analysis were taken at 14-day intervals on 8 occasions a year. The dynamics of dry matter accumulation in the whole plants and in various plant organs (stalk, leaf, grain), and that of leaf area, differed significantly between N treatments and hybrids. The effect of mineral N fertilisation was significant from the 4–6-leaf stage and the differences between hybrids from the flowering stage. Both the dry matter yield and the leaf area were greatest in the N_{160} treatment. The greatest difference in the pattern of N fertiliser reactions over time was detected between the pre-flowering and post-flowering stages. The maize grain yield was greatest in the N_{160} treatment, exhibiting the following values (t ha^{-1}) in the individual N treatments: N_0: 4.907, N_{80}: 7.872, N_{160}: 8.921, N_{240}: 8.770. The results indicate that the dynamics of dry matter accumulation in the whole maize plant and in various plant organs could further our understanding of the N fertiliser responses of maize hybrids.

Key words: maize, long-term experiment, dry matter accumulation, repeated measurements, interactions, polynomial contrasts

Introduction

Worldwide, the nitrogen use efficiency of cereal production is approximately 33%, far less than the 50% generally reported (Raun and Johnson, 1999). Production practices that have resulted in increased nitrogen use efficiency are those that counter conditions or environments known to contribute

to N loss from soil–plant systems. A common aspect of research work showing improved nitrogen use efficiency from crop rotations is the presence of excess mineral-N in the soil, and potential N loss is minimized. Differences between maize hybrids for nitrogen use efficiency are largely due to variation in the utilisation of accumulated N before anthesis, especially in the case of low N supplies (Moll et al., 1982).

Nitrogen stress is a quantitative estimate of the intensity of current nitrogen deficiency in a plant. It can be evaluated as the extent to which the growth rate of the plant falls short of the maximum growth rate attained with a non-limiting supply of nitrogen over the period when stress is being measured. The main weakness of the nutrient response curve is that it fails to deal with the changes in response that inevitably occur in the field over time. Five plant parameters have been proposed for estimating nitrogen stress – leaf nitrogen, dry weight, leaf elongation, leaf area and CER (Greenwood, 1976). Biomass accumulation is dependent on the interception of incident radiation by the crop canopy and the efficiency with which it is used to produce dry matter. The production and maintenance of leaf area are prime determinants of radiation interception. Nitrogen supply affects both leaf area development and leaf senescence. The rate of increase in leaf area should also give an accurate reflection of the influence of deficiency on growth. Furthermore, leaf area is both an expression of size and a partial expression of photosynthetic potential. Nutrition can influence photosynthesis (and hence growth) by affecting the leaf area itself or through changes in the photosynthetic rate per unit leaf area (net assimilation rate). N supply causes a greater increase in the leaf area of plants and canopies than in leaf and canopy photosynthesis (Gastal and Lemaire, 2002).

The relationship between N and biomass accumulation in crops relies on the inter-regulation of multiple crop physiological processes. Among these processes, N uptake, crop C assimilation (and thus growth rate) and C and N allocation between organs and between plants, play a particular role. Gastal and Lemaire (2002) suggested that the N uptake rate of field-grown crops was regulated not only by soil availability but also by crop growth rate. This is an important point, because crop N uptake has often been considered in relation either to soil availability (N supply approach) or to crop growth (N demand approach), and rarely to both simultaneously.

During vegetative growth, N supply had a marked influence on leaf area development in maize (Eik and Hanway, 1965; Lemcoff and Loomis, 1986; Muchow, 1988). Muchow and Davis (1988) reported that the radiation use efficiency (RUE) of maize was more responsive to N supply than was radiation interception, the latter being related to the leaf area index of the canopy. The results of Vos et al. (2005) confirmed that potato and maize show contrasting strategies to deal with N limitation. Potato adapts leaf size and avoids compromising leaf N economy and the associated photosynthetic capacity, whereas maize strives to maintain the leaf area per leaf at the expense of decreased N concentration per unit leaf area and decreased photosynthetic capacity. The importance of dry matter accumulation and allocation in genetic

improvements in maize yields was emphasised by Tollenaar et al. (1993), Ritchie and Alagarswamy (2003), Westgate et al. (2004), Berzsenyi and Lap (2005) and Nagy (2006).

Research indicates that studies on the process of dry matter accumulation over time are an ideal method for the comparative characterisation of the growth of maize plants and of the ecological and agronomic factors influencing growth (Berzsenyi and Dang, 2007; Sárvári et al., 2007). The aim of the present work was to determine the effect of N fertiliser supplies on the yield of maize hybrids using (1) the dynamics of dry matter production in the whole plant and in various plant organs (stalk, leaf, grain), and that of leaf area, and (2) the analysis of development phase × nitrogen and development phase × hybrid interactions.

Materials and methods

Treatments

The effect of mineral N fertilisation on the dry matter production of maize plants was studied in a small-plot long-term experiment set up by Béla Győrffy in 1961 on chernozem soil with forest residues. The cultivation of continuous maize for nearly 50 years can be considered as a stress environment. The N fertiliser treatments were as follows: 0, 80, 160 and 240 kg ha^{-1} (hereafter: N_0, N_{80}, N_{160}, N_{240}). All the treatments received 160 kg ha^{-1} each of P and K fertiliser. The experiment was set up in a split-plot design with four replications, with N treatments in the main plots and maize hybrids in the subplots. The investigations were carried out in 2001 and 2002 on three hybrids with different vegetation periods: Mv TC 272 (FAO 280), Mv 355 SC (FAO 390) and Maraton SC (FAO 450). The seed was sown using a Wintersteiger seed drill with row and plant spacings of 70 and 20 cm. The usual cultivation techniques were applied, with medium deep ploughing in autumn.

The rainfall quantities during the vegetation period (Apr.–Sep.) were 266 mm in 2001 and 326 mm in 2002. Although there was 60 mm more rainfall in 2002, the rainfall distribution during the flowering stage was less favourable than in 2001. From the middle of June to early July there was only 20 mm rain in 2002, compared with 53 mm in 2001. The mean temperature during the vegetation period was higher in 2002 than in 2001 (18.4 vs. 17.9°C) and there were considerably more very hot days (> 30°C) during the whole vegetation period (43 vs. 34) and in the months of June and July (29 vs. 15).

Sampling and measurements

Plant sampling for the analysis of dry matter accumulation was commenced on the 28th–35th day after sowing (in the 4-leaf stage) and continued every 14 days until physiological maturity. On each experimental plot 5 plants were cut off at ground level in the early stages and 3 plants from the 4th sampling onwards. This gave a total of 8 samplings in both years. The leaf area per plant was determined using a Delta-T laboratory leaf area meter by recording the area of each leaf, followed by summing. The dry mass of the plants was determined after drying in a drying cabinet at 90°C for 72 hours. Two rows in each plot were set aside for the determination of grain yield per treatment (t ha^{-1}). The grain yield is given for a moisture content of 15.5%.

Analysis of variance

As the experiment was set up in a split-plot design, the effect of the treatments (N fertilisation, hybrid) on the dry matter accumulation of the whole plant and of various plant organs (stalk, leaf, grain) and on the leaf area per plant was evaluated by two-way analysis of variance, for each sampling time in each year, to determine the significance of treatment effects and interactions.

Data from samples repeated over time (dry mass, leaf area) were evaluated using the GenStat 11 program for the analysis of variance for repeated measurements (Payne, 2007). The AREPMEASURES procedure calculates and applies the Greenhouse-Geisser epsilon adjustment factor for the degrees of freedom in order to examine the experimental treatment × sampling date interaction, i.e. changes in the experimental treatments over time. The significance of the linear, quadratic and third degree components of the treatments was analysed using the polynomial contrasts method. The sampling date × N fertilisation and sampling date × hybrid interactions were analysed by dissecting the SS values using the orthogonal polynomials method, based on Sváb (1973) and Gomez and Gomez (1984).

Results and discussion

The effect of N fertilisation on the total dry matter production per plant is illustrated in Figure 1, together with differences between the dry matter accumulation of individual hybrids at various sampling dates in 2001 and 2002. In all the treatments the growth dynamics exhibited a sigmoid curve. N fertilisation had a significant effect on the total dry matter production per plant, at all the sampling dates in 2001 and from the 3rd sampling date in 2002 (Table 1). In both years the curve for the N_0 treatment gradually fell behind those of the other N treatments, resulting in the significantly lowest dry matter production throughout the growth period, with final dry matter yields of 195.3 g plant^{-1} in 2001 and 141.3 g plant^{-1} in 2002 (Fig. 1). In the N_{80} treatment the dry matter production was significantly lower than in the N_{160} treatment from the 3rd sampling date in 2001 and from the 5th sampling date in 2002, with final values of 259.6 g plant^{-1} in 2001 and 222.9 g plant^{-1} in 2002. Over most of the vegetation period the dry matter production was significantly higher in the N_{160} and N_{240} treatments, with maximum values of 321.5 and 303.8 g plant^{-1}, respectively, in 2001 and 240.5 and 232.2 g plant^{-1}, respectively, in 2002 (Table 1). The dry matter accumulation of the hybrids did not differ up to flowering (until the 78th or 80th day from sowing, respectively), while the difference was significant at the last two sampling dates, when the dry matter yield of Mv 272 was significantly exceeded by that of Mv 355 and Maraton in 2001 and by that of Maraton in 2002. The nitrogen × hybrid interaction was not significant. The dynamics of dry matter accumulation exhibited a similar tendency in both years, but due to the more favourable rainfall supplies in 2001 the dry matter yield was greater than in 2002 (Table 1).

The MS values obtained using analysis of variance for repeated measurements indicated that the effect of sampling date was seven times that of N fertilisation, which was 6–7 times greater than that of the hybrid. In both years the sampling date (T) × nitrogen (N) and the sampling date (T) × hybrid (H) interactions were significant, i.e. the N response and the hybrid response varied as a function of the sampling date. The polynomial contrasts method revealed that linear effects were dominant in the N fertiliser effect and the N × T interaction, being 3–5 times as great as the quadratic effects. Only the linear

component was significant in the hybrid effect and the H × T interaction (Table 2). When the T × N and T × H interactions were dissected, the greatest contrast was found between the pre-flowering and post-flowering stages (66–68% of T × N SS and 38–50% of T × H SS).

The dynamics of dry matter accumulation in maize stalks is illustrated in Figure 2, which shows that the dry matter production increased steeply until flowering (until the 78th or 80th day from sowing, respectively), after which there tended to be no further growth, though considerable fluctuations were observed. It is interesting to note that the dynamics of maize stalk growth differs from that of total dry matter and of grain growth, which are characterised by continuous growth throughout the vegetation period. The stalk dry matter was smallest in the N_0 treatment in both years (Table 3), while the dry matter accumulation in the N_{80} treatment differed very little, if at all, from that of the N_{160} and N_{240} treatments. The final stalk mass in the two years amounted to 37.2 and 49.0 g plant^{-1} in the N_0 treatment and varied from 51.9 to 71.4 g plant^{-1} in the other treatments. Among the hybrids, the stalk dry matter accumulation was greatest for Maraton, while there was no significant difference between that of Mv 272 and Mv 355. Due to the year effect the stalk mass was smaller in 2002 than in 2001. The dynamics of leaf dry matter production was similar to that of the stalk (data not shown).

Table 1

Effect of N fertilisation and maize hybrid on the total dry matter accumulation (g plant^{-1}) at different sampling times in 2001 and 2002

Treatments	Sampling time (days from planting)							
2001	38	52	66	80	94	108	122	136
N_0	3.0b	12.4c	33.0b	76.0b	106.3c	149.8b	155.1c	195.3c
N_{80}	3.2b	14.6bc	50.3a	97.5ab	134.7b	187.1ab	236.3b	259.6b
N_{160}	4.6a	20.0ab	58.0a	110.7a	163.9a	221.4a	288.8a	321.5a
N_{240}	5.3a	23.1a	59.1a	113.9a	153.4a	216.1a	286.3a	303.8a
H_1	4.1a	18.6a	50.9a	94.5a	133.7a	179.2a	222.3b	233.3b
H_2	3.9a	17.0a	51.6a	101.9a	147.3a	194.1a	252.6a	282.8a
H_3	4.1a	17.7a	47.8a	102.2a	137.6a	207.5a	250.0a	294.0a
2002	36	50	64	78	92	106	120	134
N_0	2.1a	14.5a	38.4b	74.8b	100.4c	129.9c	143.1c	141.3b
N_{80}	2.4a	23.0a	50.8ab	95.6a	124.7b	166.4b	200.9b	222.9a
N_{160}	3.2a	20.5a	55.1a	107.7a	149.3a	198.2a	237.5a	240.5a
N_{240}	2.6a	22.3a	56.9a	98.5a	125.8b	190.3a	237.5a	232.2a
H_1	2.9a	20.3a	54.2a	88.1a	131.0a	170.3a	189.4b	185.2b
H_2	2.4b	19.7a	48.6a	98.9a	113.9a	167.2a	201.4ab	206.8b
H_3	2.4b	20.3a	48.1a	95.5a	130.2a	176.2a	223.6a	235.6a

Means followed by the same letter within a treatment group and sampling time are not significantly different according to Duncan's multiple range test ($P \leq 0.05$)

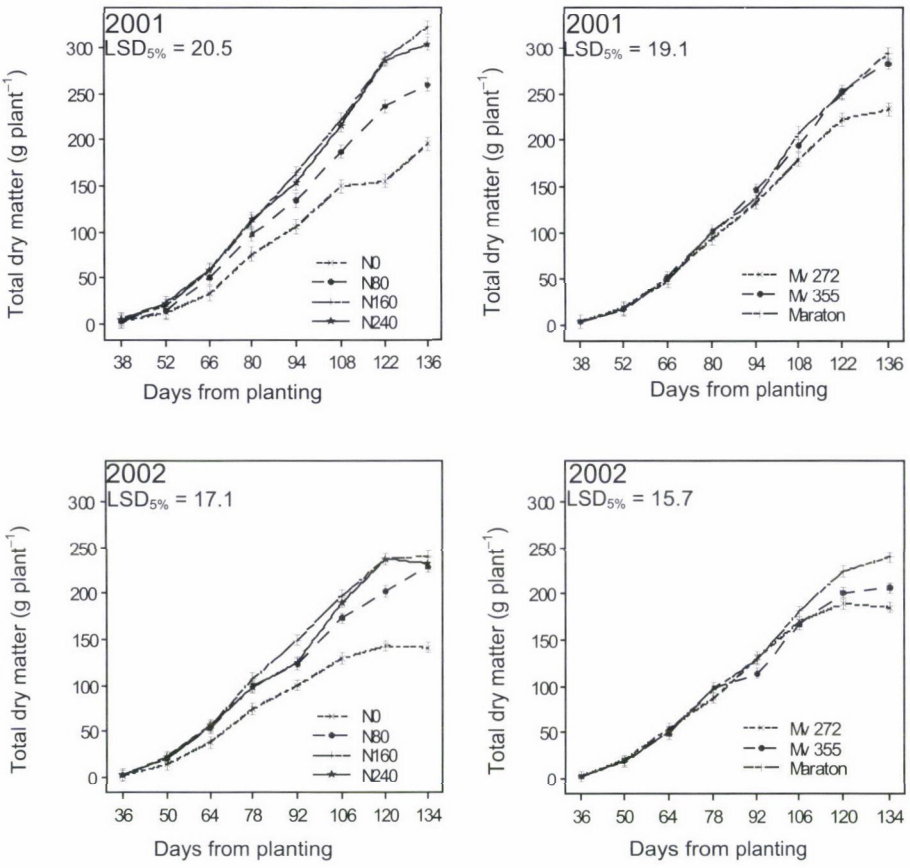

Fig. 1. Seasonal dynamics of total dry matter accumulation as a function of N fertilisation and maize hybrid, based on measured data in 2001 and 2002. Vertical bars indicate the standard errors of the means. LSD values for the interactions are presented

The MS values obtained using analysis of variance for repeated measurements indicated that the effect of sampling date was 7–10 times that of N fertilisation and hybrid. The polynomial contrasts method indicated that linear and quadratic effects were significant in the N fertiliser effect and the N × T interaction (60–80% linear effect and 20–40% quadratic effect, based on SS). Linear and quadratic components were also significant in the hybrid effect and the H × T interaction in 2001, though the linear effect made up 90% of SS for the hybrid and 74% for H × T. In 2002 only the linear effect was significant (Table 2). Dissection of the T × N and T × H interactions revealed the greatest contrast between the pre-flowering and post-flowering stages, amounting to 40–83% of SS for T × N and 55–60% for T × H.

Table 2

Mean square (MS) values of analysis of variance on repeated measurements on different maize plant characteristics in 2001 and 2002

Variance component	df[a]	Total dry matter	Stalk dry matter	Leaf area	Grain dry matter	Total dry matter	Stalk dry matter	Leaf area	Grain dry matter
		2001				2002			
Nitrogen (N)	3	140.3***	46.7***	71.5***	100.7***	76.2***	19.6***	34.2***	50.3***
Lin	1	353.3***	112.1***	167.1***	259.8***	162.6***	35.6***	84.7***	112.0***
Quad	1	62.1***	25.2***	46.4***	41.3****	65.9***	23.0***	17.7**	38.9***
Cub	1	5.5*	2.9^{NS}	<1	<1	<1	<1	<1	<1
Hybrid (H)	2	7.6**	26.6***	39.5***	4.7*	6.1**	39.9***	69.4***	1.6^{NS}
Lin	1	12.2**	52.3***	78.9***	<1	10.6**	71.5***	133.8***	<1
Quad	1	3.0^{NS}	<1	<1	8.7**	1.5^{NS}	8.2**	5.1*	3.0^{NS}
Time (T)	7 (4)	730.8***	479.9***	297.7***	730.0***	731.6***	572.1***	542.7***	635.6***
N × H	6	2.4^{NS}	2.0^{NS}	3.5*	3.0*	<1	<1	2.2^{NS}	<1
N × T	21 (12)	9.4***	2.8***	3.2***	14.8***	9.4***	3.3***	9.4***	12.1***
Lin	7 (4)	22.6***	5.3***	4.0***	35.0***	19.9***	5.1***	21.5***	26.4***
Quad	7 (4)	5.0***	2.5*	4.4***	9.2***	7.0***	3.9***	4.8***	9.0***
H × T	14 (8)	3.4***	6.7***	6.9***	6.0***	4.6***	9.0***	13.5***	7.7***
Lin	7	5.8***	11.2***	13.3***	8.3***	8.1***	13.2***	25.7***	13.0***
Quad	7	1.0^{NS}	2.1*	<1	3.8**	1.2^{NS}	4.7***	1.2^{NS}	2.4^{NS}
N × H × T	42 (21)	1.8**	<1	1.2^{NS}	1.8***	<1	<1	1.3^{NS}	<1

Figures in brackets are the df values for grain yield, where these differed from the others. ***, **, * Significant at the P = 0.1%, P = 1% and P = 5% levels, respectively; ^{NS} = non-significant.

Table 3

Effect of N fertilisation and maize hybrid on the stalk dry matter accumulation (g plant^{-1}) at different sampling times in 2001 and 2002

Treatments	Sampling time (days from planting)							
2001	38	52	66	80	94	108	122	136
N_0	1.0b	4.7c	15.6b	47.2b	47.9c	47.5b	41.1b	49.0c
N_{80}	1.0b	5.6bc	26.2a	53.1ab	57.7b	57.1a	55.1a	60.2b
N_{160}	1.7a	8.1ab	30.6a	63.7a	68.1a	62.3a	63.0a	71.4a
N_{240}	1.9a	9.6a	31.1a	64.6a	63.3ab	58.6a	62.2a	64.0ab
H_1	1.4a	7.4a	27.8a	49.0b	49.9b	45.1a	48.4c	55.7b
H_2	1.4a	7.0a	26.5a	60.4a	63.9a	57.0a	54.4b	58.4b
H_3	1.5a	6.6a	23.3a	61.9a	64.0a	66.9a	63.2a	69.4a
2002	36	50	64	78	92	106	120	134
N_0	0.5b	5.7b	18.8b	45.7b	47.5b	43.9b	37.4c	37.2b
N_{80}	0.7ab	8.4ab	28.6a	57.8a	55.0ab	52.7a	46.2b	51.9a
N_{160}	0.9a	8.5ab	29.4a	62.3a	61.0a	55.4a	52.9a	55.5a
N_{240}	0.8ab	9.1a	30.0a	55.9a	50.5b	55.7a	52.4a	52.1a
H_1	0.8a	8.4a	29.5a	47.9b	48.1b	45.7c	42.7b	44.2b
H_2	0.7a	8.3a	25.3b	58.6a	50.5b	50.7b	42.4b	43.8b
H_3	0.7a	7.1a	25.4b	59.8a	61.8a	59.3a	56.6a	59.5a

Means followed by the same letter within a treatment group and sampling time are not significantly different according to Duncan's multiple range test ($P \leq 0.05$)

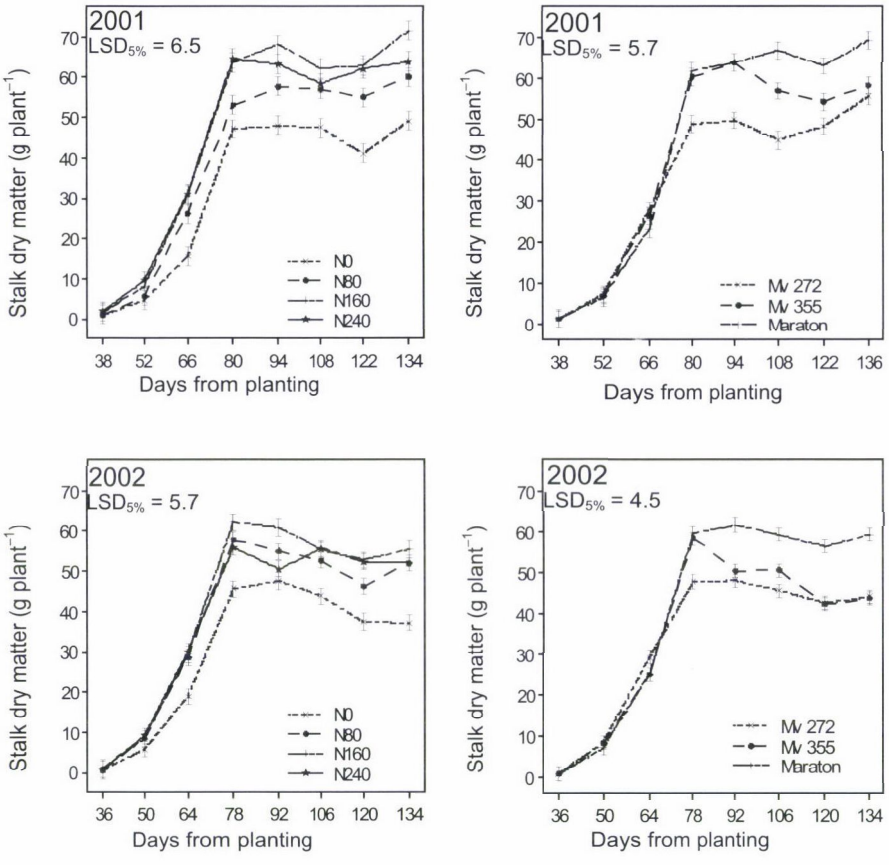

Fig. 2. Seasonal dynamics of stalk dry matter accumulation as a function of N fertilisation and maize hybrid, based on measured data in 2001 and 2002. Vertical bars indicate the standard errors of the means. LSD values for the interactions are presented

The seasonal dynamics of leaf area per plant could be described by a parabola (Fig. 3). The leaf area was significantly the smallest in plots without N fertiliser (N_0) from the 1st or 2nd measurement date. It is clear from Figure 3 that the N_0 curve rapidly fell behind that of the other treatments in both years. The maximum leaf area per plant in the N_0 treatment was 3721 cm² in 2001 and 3921 cm² in 2002 (Table 4). In response to N fertilisation there was a significant increase in leaf area, though the values for the N_{80}, N_{160} and N_{240} treatments did not differ significantly at the first four measurement dates. After this the leaf area in the N_{80} treatment was significantly smaller than that of the other treatments, particularly in 2002. The difference between the N_{160} and N_{240} treatments varied at each measurement date, but in both years the greatest leaf area was recorded in the N_{160} treatment (5398 cm² in 2001 and 5138 cm² in

2002). After flowering the leaf area declined steeply in the N_0 treatment, while it remained constant for 3–4 weeks in the other treatments, thereafter decreasing to different extents at each N rate. No differences in leaf area were observed between the hybrids up to the 4th measurement date, after which the differences became significant. In both years the leaf area per plant exhibited a close correlation with the maturity group, with the greatest leaf area for Maraton (4983 cm^2 in 2001, 5558 cm^2 in 2002), followed by Mv 355 (4789 and 4669 cm^2, respectively) and Mv 272 (3953 and 4260 cm^2, respectively).

The MS values obtained using analysis of variance for repeated measurements on leaf area indicated that the effect of sampling date was more than three times that of N fertilisation, while that of N fertilisation and hybrid were

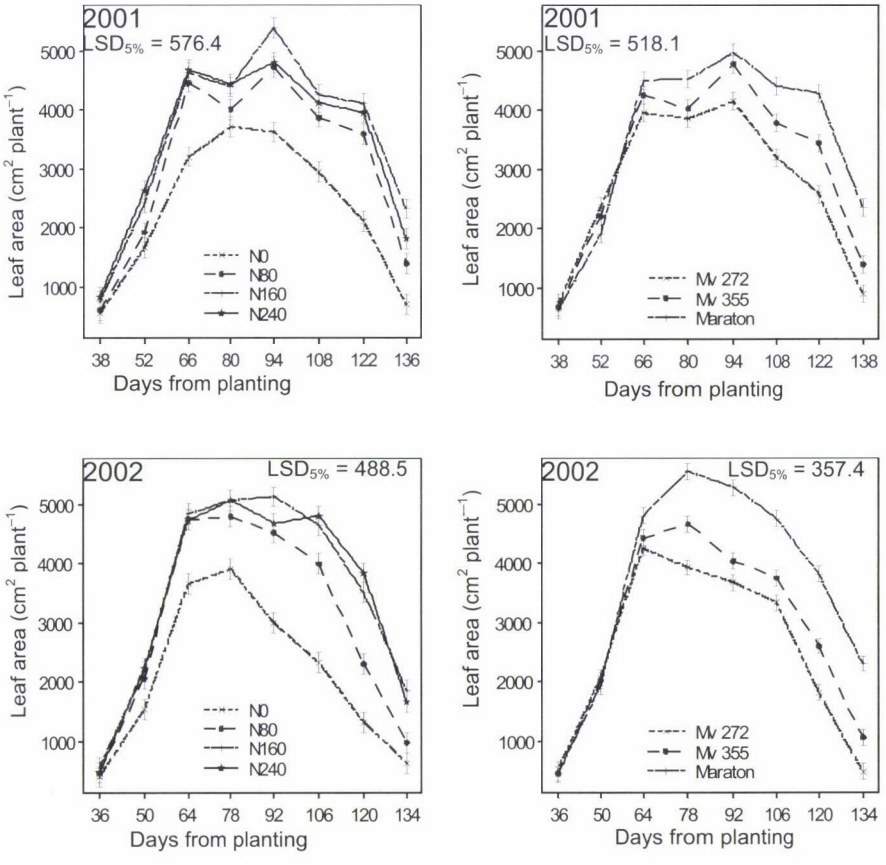

Fig. 3. Seasonal dynamics of leaf area as a function of N fertilisation and maize hybrid, based on measured data in 2001 and 2002. Vertical bars indicate the standard errors of the means. LSD values for the interactions are presented

Table 4
Effect of N fertilisation and maize hybrid on the leaf area per plant (cm^2) at different sampling times in 2001 and 2002

Treatments	Sampling time (days from planting)							
2001	38	52	66	80	94	108	122	136
N_0	547b	1667b	3206b	3721b	3366b	2945c	2112c	692c
N_{80}	601b	1923b	4468a	4016ab	4725a	3872b	3596b	1395bc
N_{160}	741ab	2423a	4621a	4398a	5398a	4259a	4107a	2318a
N_{240}	821a	2640a	4675a	4447a	4812a	4125ab	3956ab	1809ab
H_1	737a	2369a	3953b	3868b	3953b	3206c	2589c	896b
H_2	670ab	2210a	4259ab	4031b	4789a	3785b	3452b	1397b
H_3	626a	1910b	4515a	4536a	4983a	4411a	4287a	2368a
2002	36	50	64	78	92	106	120	134
N_0	410b	1537b	3664b	3921b	2998b	2332c	1323c	606c
N_{80}	463ab	2064ab	4750a	4802a	4527a	4002b	2305b	979bc
N_{160}	560a	2190a	4856a	5079a	5138a	4661a	3676a	1828a
N_{240}	468ab	2241a	4744a	5081a	4688a	4814a	3850a	1662ab
H_1	528a	2068a	4260b	3936c	3682b	3338b	1935c	423c
H_2	446b	2027a	4439b	4669b	4041b	3754b	2604b	1061b
H_3	451b	1930a	4812a	5558a	5290a	4765a	3826a	2323a

Means followed by the same letter within a treatment group and sampling time are not significantly different according to Duncan's multiple range test ($P \leq 0.05$)

almost the same. In the case of the N fertiliser effect and the N × T interaction, the polynomial contrasts method showed the dominance of the linear effect, which was 3–5 times greater than the quadratic component. For the hybrid effect the linear component was significant in 2001 and both the linear and the quadratic component in 2002 (Table 2). Dissection of the T × N and T × H interactions revealed the greatest contrast between the pre-flowering and the post-flowering stages, amounting to 46–53% of SS for T × N and 59–64% for T × H.

The dynamics of dry matter accumulation in the grain, which was similar to that of total dry matter per plant, is illustrated in Figure 4 as a function of N fertiliser and hybrid. The greatest dry matter accumulation was recorded in the N_{160} and N_{240} treatments, with values of 169.4 and 163.7 g $plant^{-1}$ in 2001 and 121.1 and 119.3 g $plant^{-1}$ in 2002, respectively (Table 5). The dry matter accumulation in the N_0 treatment was significantly smaller than in the other treatments from the 108th day after sowing in 2001 and from the 76th day in 2002. At physiological maturity the grain dry matter accumulation in the N_0 treatment was 91.4 g $plant^{-1}$ in 2001 and 61.5 g $plant^{-1}$ in 2002, which was approximately half the values recorded in the N_{160} and N_{240} treatments. The dry matter accumulation in the N_{80} treatment was only significantly smaller than that in the N_{160} and N_{240} treatments during part of the grain-filling period. In both years the dry matter accumulation of the Mv 272 hybrid was significantly greater in the early stages of grain filling, after which it gradually declined and became the smallest of all the hybrids during the final stages of grain filling. The greatest grain dry mass was recorded for Mv 355 and Maraton, with values of 154.5 and 150.3 g $plant^{-1}$, respectively, in 2001 and 111.1 and 114.6 g $plant^{-1}$ in 2002. A comparison of the two years shows that dry matter incorporation into the grain was greater in

2001. In the less favourable year of 2002 dry matter accumulation was terminated earlier, on the 120th day, in the N_0, N_{160} and N_{240} treatments.

The MS values obtained using analysis of variance for repeated measurements on grain filling indicated that the effect of sampling date was 7–9 times greater than the N fertiliser effect, while the hybrid effect was considerably smaller than the N fertiliser effect. Based on polynomial contrasts, linear and quadratic effects were significant in the N fertiliser effect and the N × T interaction (72–86% SS linear, 14–28% SS quadratic). The quadratic effect was significant in the hybrid effect in 2001, while in 2002 the hybrid effect was not significant. For the H × T interaction, the linear and quadratic components were significant in 2001 and the linear component in 2002 (Table 2). Dissection of the T × N and T × H interactions revealed the greatest contrast between the initial phase of grain filling and the last two sampling dates, amounting to 88–92% of T × N SS and 62–68% of T × H SS. In the case of the T × H interaction the contrast between the initial and final stages of grain filling and the middle stage was also significant (24–26% of T × H SS).

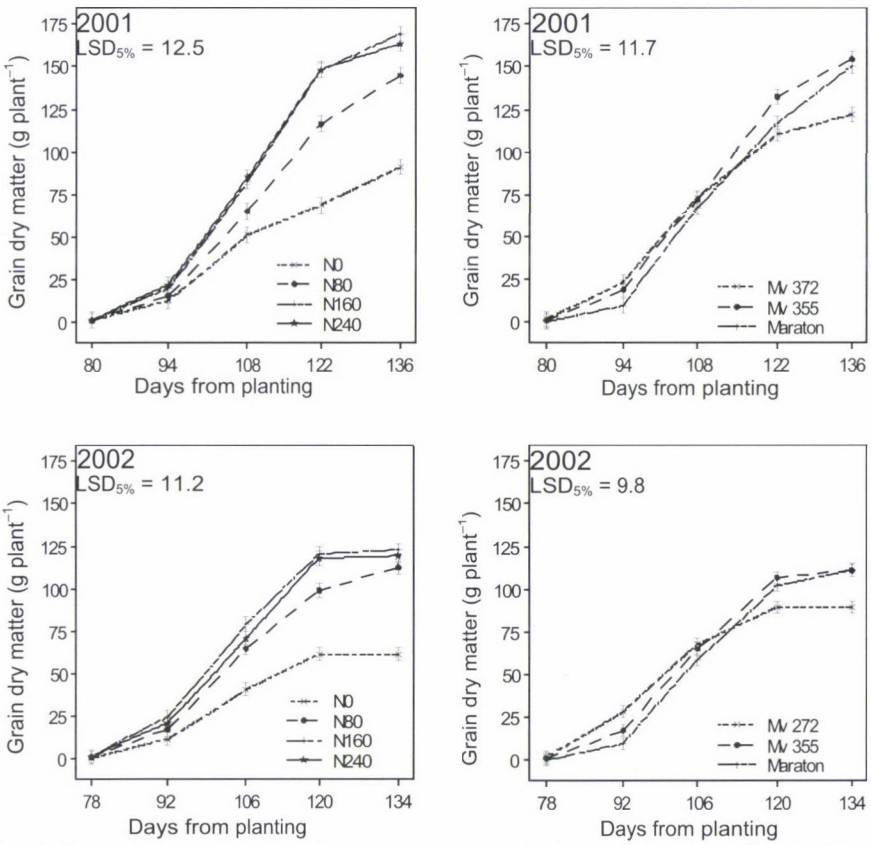

Fig. 4. Seasonal dynamics of grain dry matter accumulation as a function of N fertilisation and maize hybrid, based on measured data in 2001 and 2002. Vertical bars indicate the standard errors of the means. LSD values for the interactions are presented

Table 5
Effect of N fertilisation and maize hybrid on the grain dry matter accumulation (g plant^{-1}) at different sampling times in 2001 and 2002

Treatments	Sampling time (days from planting)									
	80	94	108	122	136	78	92	106	120	134
	2001					2002				
N_0	0.79a	13.0a	51.6b	68.7c	91.4c	0.66b	9.8b	42.6c	61.6b	61.5b
N_{80}	1.35a	15.8a	65.1ab	116.4b	144.8b	1.31a	18.8a	65.0b	99.0a	116.6a
N_{160}	1.42a	18.9a	82.6a	147.7a	169.4a	1.63a	24.6a	79.2a	120.4a	121.1a
N_{240}	1.49a	21.7a	85.2a	148.2a	163.7ab	1.58a	21.0a	72.7ab	117.9a	119.3a
H_1	2.17a	23.2a	73.6a	110.7b	122.1b	2.31a	27.8a	69.2a	89.8a	88.3b
H_2	1.31b	18.7a	72.2a	132.6a	154.5a	1.29b	18.0b	66.4a	107.0a	111.1a
H_3	0.48c	10.1b	67.5a	117.ab	150.3a	0.28c	10.0c	59.0a	102.2a	114.5a

Means followed by the same letter within a treatment group, sampling time and year are not significantly different according to Duncan's multiple range test ($P \leq 0.05$)

In both years N fertilisation had a significant effect (P = 0.1%) on the grain yield per hectare, while the difference between the hybrids was significant at the P = 0.1% level in 2001 and at the P = 1% level in 2002 (Fig. 5). The N fertiliser × hybrid interaction was not significant in either year. In response to N fertilisation there was a significant increase in grain yield up to the N_{160} rate in 2001 and up to the N_{80} rate in 2002. The grain yields in each N treatment and year were the following (t ha^{-1}): in 2001: N_0: 5.603, N_{80}: 8.618, N_{160}: 9.654, N_{240}: 9.774; in 2002: N_0: 4.211, N_{80}: 7.126, N_{160}: 8.188, N_{240}: 7.766. In 2001 the grain yield rose significantly with the length of the vegetation period (t ha^{-1}): Mv 272: 7.129, Mv 355: 8.551, Maraton: 9.557, while in 2002, due to the unfavourable weather, the yields were lower and that of Maraton was significantly lower than that of Mv 355 (t ha^{-1}): Mv 272: 6.792, Mv 355: 7.299, Maraton: 6.377.

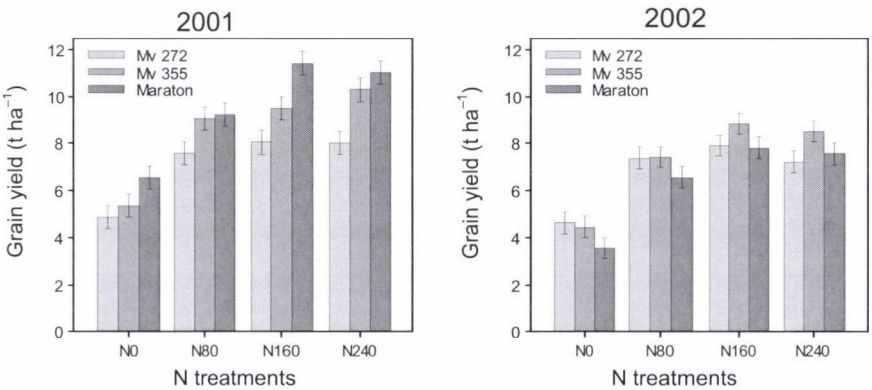

Fig. 5. Effect of N fertilisation on the grain yield of different maize hybrids in 2001 and 2002. Vertical bars indicate the standard errors of the means

Conclusions

N fertilisation was found to have a significant effect on the growth dynamics of dry matter accumulation in both the whole maize plant and the various plant organs (leaf, stalk, grain) and on that of the leaf area. Various physiological and morphological traits have been proposed as good descriptors of the response of maize to N availability. The dry matter accumulation in the whole plant and the grain (and also that of the maize ear) exhibited great similarity and could be described with a sigmoid curve. By contrast, the dry matter accumulation in the stalk (and the leaves) was initially characterised by a steep linear increase, while after flowering this growth trend was not continued, though considerable fluctuations were observed. The seasonal dynamics of leaf area per plant could be depicted as a parabola.

For all the traits the effect of N fertiliser treatments was significant right from the first measurement date, while differences between the hybrids became significant later, from the second half of the generative phase. The curve of the N_0 treatment was the first to fall behind the other treatments, followed 14–21 days later by that of the N_{80} treatment. The greatest values of dry matter production and leaf area were recorded in the N_{160} treatment. The greatest differences in the sampling date × N treatment interaction were observed between the vegetative and generative phases and between the initial and final stages of grain filling. The dynamics of dry matter accumulation (and leaf area) over time was influenced to the greatest extent by the sampling date, followed by N fertilisation, while the hybrid had the smallest effect. Both linear and quadratic components were significant in the N fertiliser effect, but the linear effect was much the strongest. In most cases the linear component was dominant in the hybrid effect.

It can be concluded that, although the seasonal dynamics of the traits investigated could be described with different types of functions, they gave similar values for the effects of N fertiliser and hybrid and for the level of N stress. The change in stalk dry weight from silking to physiological maturity is an indicator of the ratio of assimilate demand by the grain and assimilate supply by the leaf canopy during the post-silking period (Rajcan and Tollenaar, 1999). The experimental data demonstrated the year effect, which influenced the dynamics of the N fertiliser response of the hybrids. The results suggest that the dynamics of dry matter accumulation in the whole plant and in individual plant organs may contribute to our understanding of changes in the N fertiliser response over time and to improving the N utilisation of maize hybrids. A further paper will discuss the use of growth analysis to describe the N fertiliser responses of maize hybrids and the extent of N stress.

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WEED SHIFT IN A MAIZE (*Zea mays* L.) – SUNFLOWER (*Helianthus annuus* L.) CROPPING SYSTEM

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A field experiment was conducted during the *kharif* (June–September) and *rabi* (October–January) seasons of 2005–2006 to study the effect of a maize – sunflower cropping system on the weed flora shift. The results revealed a change in weed species, i.e. the appearance of new species and the elimination of certain weed species due to the cropping system. The density of *Dinebra retroflexa* was high during the 1st year maize cropping period, but *Panicum repens* became dominant when sunflower was grown after maize. *Cyperus rotundus*, originally the dominant sedge, was smothered by *Cynodon dactylon* due to zero tillage. *Dactyloctenium aegyptium* was the dominant weed species in maize, while *Parthenium hysterophorus* was the dominant weed species in sunflower. The proportions of *Datura fastuosa, Parthenium hysterophorus, Trianthema portulacastrum, Amaranthus viridis, Amaranthus polygamus, Flaveria austerlagica, Gynandropsis pentaphylla* and *Portulaca quadrifida* were higher during the 1st year maize cropping season, while later their density was gradually reduced due to the inclusion of sunflower in the system.

Key words: maize, sunflower, weed shift, relative density, cropping system

Introduction

Weeds constitute a major constraint to successful crop production. The cropping system influences the weed intensity and weed flora composition in cultivated fields. Differences in the weed communities between crops can also be expected when crops with different life cycles are grown. Certain weeds are closely associated with a particular crop or cropping system. A cropping system may introduce conditions that are not favourable for a specific weed species and thus the growth and reproduction of that species are hampered. Growing maize in rotation with cotton (*Gossypium hirsutum*) effectively controlled the infestation with Johnson grass (*Sorghum halepense*) in a maize – cotton – cotton – maize cropping system (Dale and Chandler, 1979).

Continuous cropping produced the greatest weed densities of green foxtail (*Setaria viridis*), thyme-leaved spurge (*Euphorbia serpyllifolia* Pers.) and vetch (*Vicia sativa*). In most instances, Canada thistle (*Cirsium arvense*) was the most abundant weed on continuously cropped fields (Hume, 1982). The green foxtail and wild oat (*Avena fatua*) densities increased in continuous mono-cropping of wheat (Fay, 1990). Biswas and Das (1993) reported a shift in the weed flora from annuals to perennials (*Cyperus rotundus*) in jute (*Corchorus olitorius*) when jute was rotated with a rice (*Oryza sativa*) – wheat (*Triticum aestivum*) system. Saikia and Pandey (1999) observed a weed flora shift from dicots like *Trianthema portulacastrum* in the first year to monocots like *Digitaria sanguinalis* in the second year of a maize – chickpea cropping system. Crop rotation may disrupt the continuous dominance of a specific weed in a field, decrease the buildup of the weed population, and prevent major shifts in weed species composition.

Materials and methods

A field experiment was conducted at Tamil Nadu Agricultural University, Coimbatore during the *Kharif* (June–Sept.) and *Rabi* (Oct.–Jan.) seasons of 2005 and 2006 to study the effect of tillage and weed management methods on the weed flora shift in a maize – sunflower cropping system. The soil was clay loam in texture and the available N, P and K contents of the soil were 325.5, 12.5 and 365.4 kg ha^{-1}, respectively. The experiment was laid out in a split plot design with four replications. The main plot treatment consisted of four tillage methods, namely zero tillage – zero tillage (T_1), zero tillage – conventional tillage (T_2), conventional tillage – zero tillage (T_3) and conventional tillage – conventional tillage (T_4). The subplot treatments consisted of three weed management methods, namely hand weeding at 20 and 40 days after sowing (DAS) (W_1), pre-emergence herbicide application (atrazine 0.5 kg ha^{-1} for maize and pendimethalin 1.0 kg ha^{-1} for sunflower) followed by hand weeding at 40 DAS (W_2), and an unweeded check for both the crops (W_3).

Maize variety CO-1, with a field duration of 105–110 days, and sunflower variety CO-4, with a field duration of 85–90 days, were selected for the study. In zero tillage the seeds were dibbled in the stubble of the previous crop without any tillage or soil disturbance. For conventional tillage one mould board ploughing / disc ploughing was given as the primary tillage operation, followed by secondary tillage with a disc harrow. Weed management was done as per the treatment schedule.

Observations on weed flora, weed density and weed dry weight were made at 20 DAS in both the crops. The predominant weed species in the unweeded control were observed and grouped as grasses, sedges and broad-leaved weeds. The weed density in each plot was recorded using a quadrate (0.5 × 0.5 m) in four places at random and expressed as number m^{-2} (Burnside and Wicks, 1965). The predominant grasses, sedges and broad-leaved weeds in each plot were recorded separately and expressed in No. m^{-2}. The relative density of the individual predominant weed species and groups of weeds was calculated as detailed below and expressed as a percentage.

$$\text{Relative density (RD \%)} = \frac{\text{Absolute density of a given species (No. m}^{-2})}{\text{Total absolute density of all species (No. m}^{-2})} \times 100$$

Results and discussion

The appearance of new weed species and the elimination of certain weed species were observed due to the use of a maize – sunflower cropping system (Table 1). The list of new weed species recorded during the experimental period of two years revealed that these weeds included two species of monocots and eleven species of dicots. Out of these, five broad-leaved weeds and one grass weed which were present initially disappeared at later stages. Other species were present at different periods of the experimentation.

The broad-leaved weeds *Abutilon indicum, Aerva lanata, Aeschynomone indica, Cassia nigricans* and *Hibiscus vitifolius* and the grass weed *Cyanotis cucullata* were new weeds during the first year of maize. Weeds such as *Phyllanthus amarus, Trichodesma indicum, Lagasca mollis, Rynchosia minima, Tribulus terrestris* and *Eclipta alba* were found during the second year of maize, while *Digitaria bicornis* was observed in first year sunflower.

Dinebra retroflexa was the dominant weed species during the initial period of the cropping system, i.e. in the first maize crop (Table 2). Afterwards, due to the inclusion of sunflower in the system, its density was gradually reduced, irrespective of tillage and weed management practices. The dominance of *Dinebra retroflexa* in the sunflower growth period was smothered by *Panicum repens*, so the *Panicum repens* density was higher in the sunflower crop period. The density of *Dinebra retroflexa* was reduced from 13.1 m^{-2} to 1.8 m^{-2} when maize was grown in the 2nd year. Similar results were observed by Derksen et al. (1995) and Tingle and Chandler (2004). Saikia and Pandey (1999) reported a weed flora shift from dicots like *Trianthema portulacastrum* in the first year to monocots like *Digitaria sanguinalis* in the second year during the rainy season in a maize – chickpea cropping system. In a sorghum – cotton cropping system Ponnuswamy and Kandasamy (1996) observed the dominance of *Trianthema portulacastrum* and *Echinochloa colonum* in the first crop of sorghum and *Chloris barbata* in the subsequent cotton crop.

In general the density of *Panicum repens* was higher in the early stages of sunflower and thereafter the density decreased with the age of the crop, as *Panicum repens* matured earlier than sunflower. During the second year, because of the rotation of sunflower with maize, the density of *Panicum repens* was reduced from 14.9 to 3.6 m^{-2}, but the density of *Dactyloctenium aegyptium* (Table 2) was higher in maize than in sunflower. The density of *Parthenium hysterophorus* was higher in sunflower than maize. but its density was reduced when the crop was rotated with maize. So the density of *Parthenium hysterophorus* during second year sunflower was low compared to first year sunflower.

The density of *Digera arvensis* was greater in the 1st year maize crop compared to subsequent sunflower, but during the 2nd year its density was lower in maize than in sunflower. Crop rotation was obviously unfavourable for this weed species. The density of *Datura fastuosa* was higher during 1st year maize,

Table 1
Weed flora dynamics in a maize–sunflower cropping system

Weed species	General weed species	1st year		2nd year	
		Maize	Sunflower	Maize	Sunflower
A. Grasses					
**Chloris barbata* Swasts	√	√	√	√	√
Cyanotis cucullata	X	√	X	X	X
**Cynodon dactylon*	√	√	√	√	√
**Dactyloctenium aegyptium*	√	√	√	√	√
**Dinebra retroflexa*	√	√	√	√	√
Digeria longiflora	√	√	√	√	√
Digeria bimaizeis	X	X	√	X	X
Echinochloa colonam	√	√	√	√	√
**Panicum repens* L.	√	√	√	√	√
Panicum flavidum	√	√	√	√	√
Pennisetunm cenchroides	√	√	√	√	X
Rotabella cochinsinensis	√	√	√	√	√
Saccolepis interupta	√	√	√	X	√
Setaria verticiliata	√	√	√	√	X
B. Sedges					
**Cyperus rotundus* L.	√	√	√	√	√
C. Broad-leaved weeds					
Abutilon indicum	X	√	X	X	X
Acalypha indica	√	√	√	√	√
Aerva lanata	X	√	X	X	X
Aeschynomene indica	X	√	X	X	X
Amaranthus polygamus	√	√	√	√	√
Amaranthus viridis	√	√	√	√	√
Aristolochia bracteata	√	X	X	√	√
Boerhaavia erecta	√	√	√	√	√
Cassia nigricans	X	√	X	X	X
Corchorus trilocularis	√	√	√	√	√
**Datura metel*	√	√	√	√	√
**Digera arvensis*	√	√	√	√	√
Eclipta alba	X	X	X	√	X
Euphorbia prostrata	√	√	X	√	√
Flaveria austerlagica	√	√	X	√	X
Gynandropsis pentaphylla	√	√	√	X	X
Hisbiscus vitifolius	X	√	X	X	X
Lagasca mollis	X	X	X	√	X
**Parthenium hysterophorus*	√	√	√	√	√
Phyllanthus amarus	X	X	X	√	X
Phyllanthus madaraspatensis	√	√	√	√	√
Portulaca quadrifida	√	√	√	X	X
Rynchosia minima	X	X	X	√	X
**Trianthema portulacastrum*	√	√	√	√	√
Trichodesma indicum	X	X	X	√	X
Tribulus terrestris	X	X	X	X	√

√: Weed species present, X: Weed species absent; *: Dominant weed species detailed in Table 2

Table 2
Effect of tillage and weed management practices on relative density (%) of grass, sedge and broad-leaved weeds at 20 DAS in a maize – sunflower – maize – sunflower cropping system*

Weed species	Treatments							
	T_1	T_2	T_3	T_4	W_1	W_2	W_3	Mean
Dinebra retroflexa (A)	20.1	14.8	4.2	13.1	10.4	10.1	18.7	13.1
Dinebra retroflexa (B)	5.7	4.9	2.2	2.2	2.8	5.1	3.3	3.7
Dinebra retroflexa (C)	3.0	1.8	0.0	2.8	5.7	0.0	0.0	1.9
Dinebra retroflexa (D)	4.2	1.1	1.1	0.9	1.5	0.9	3.1	1.8
Cynodon dactylon (A)	9.4	6.1	7.6	13.2	7.9	13.9	5.4	9.1
Cynodon dactylon (B)	10.2	1.4	3.4	6.3	4.8	8.3	2.9	5.3
Cynodon dactylon (C)	17.6	10.6	17.3	13.4	7.5	14.7	21.9	14.7
Cynodon dactylon (D)	33.1	13.2	17.4	17.8	11.8	34.7	20.4	14.7
Dactyloctenium aegyptium (A)	6.5	1.8	6.1	2.4	2.9	2.2	7.5	4.2
Dactyloctenium aegyptium (B)	20.5	4.3	6.6	1.9	14.1	1.4	9.6	8.4
Dactyloctenium aegyptium (C)	9.8	14.0	4.0	4.0	6.7	5.4	11.7	7.9
Dactyloctenium aegyptium (D)	6.5	4.7	5.8	4.6	6.1	5.2	4.8	5.4
Chloris barbata (A)	10.8	3.4	11.0	4.6	4.6	3.7	14.0	7.4
Chloris barbata (B)	0.0	0.3	2.6	2.5	0.0	0.0	4.1	1.4
Chloris barbata (C)	9.8	13.3	0.0	0.0	2.6	4.2	10.6	5.8
Chloris barbata (D)	10.6	4.1	8.3	0.9	2.4	4.2	11.3	6.0
Panicum repens (A)	0.2	0.2	0.5	0.6	0.1	0.4	0.6	0.4
Panicum repens (B)	5.6	16.7	10.4	27.1	24.3	2.6	17.9	14.9
Panicum repens (C)	0.8	0.8	0.5	0.9	0.8	0.7	0.9	0.8
Panicum repens (D)	6.7	4.2	3.3	0.3	4.3	3.1	3.5	3.6
Cyperus rotundus (A)	4.2	7.4	19.8	25.8	7.5	26.5	8.9	14.3
Cyperus rotundus (B)	18.4	16.8	19.5	16.9	13.9	32.7	7.0	17.9
Cyperus rotundus (C)	33.4	29.3	62.1	63.4	53.4	63.3	24.5	47.1
Cyperus rotundus (D)	3.9	19.6	8.9	23.9	9.5	27.6	5.1	14.1
Trianthema portulacastrum (A)	17.6	33.6	36.9	28.7	44.6	22.0	21.1	29.2
Trianthema portulacastrum (B)	6.1	10.1	8.1	5.8	8.1	4.8	9.6	7.5
Trianthema portulacastrum (C)	5.5	6.7	5.6	5.5	7.4	3.1	7.0	5.8
Trianthema portulacastrum (D)	7.3	16.1	15.5	15.5	18.4	2.9	19.4	13.6
Parthenium hysterophorus (A)	1.3	2.1	1.2	1.4	1.2	0.3	3.0	1.5
Parthenium hysterophorus (B)	25.1	21.2	24.8	25.4	18.4	28.9	25.0	24.1
Parthenium hysterophorus (C)	6.6	7.1	2.0	0.9	5.2	1.9	5.5	4.2
Parthenium hysterophorus (D)	10.4	16.1	13.7	17.6	20.2	6.7	16.5	14.5
Digera arvensis (A)	16.2	16.8	5.7	6.0	7.5	13.4	12.6	11.2
Digera arvensis (B)	1.2	1.7	0.4	0.0	0.9	0.0	1.5	0.8
Digera arvensis (C)	3.8	3.3	11.5	4.6	8.8	3.9	4.7	5.8
Digera arvensis (D)	3.7	5.1	4.2	5.9	6.1	3.3	4.9	4.8
Datura metel (A)	5.0	5.3	4.1	0.8	9.1	0.8	1.6	3.8
Datura metel (B)	2.0	4.1	2.6	2.5	3.3	3.0	2.2	2.8
Datura metel (C)	1.4	1.7	2.0	1.8	3.1	0.3	1.7	1.7
Datura metel (D)	1.2	2.5	4.2	2.9	3.4	1.2	3.4	2.7
Amaranthus viridis (A)	5.4	5.6	1.9	2.0	2.5	3.9	4.7	3.7
Amaranthus viridis (B)	1.8	1.2	2.1	3.0	2.1	1.3	2.6	2.0
Amaranthus viridis (C)	0.8	0.0	0.0	0.0	0.2	0.0	0.4	0.2
Amaranthus viridis (D)	0.0	0.0	0.0	0.6	0.2	0.0	0.0	0.1
Amaranthus polygamus (A)	1.8	1.9	0.6	0.7	0.8	1.3	1.6	1.2
Amaranthus polygamus (B)	3.3	2.4	0.7	0.5	0.6	1.2	3.3	1.7
Amaranthus polygamus (C)	0.0	0.0	1.3	1.0	0.4	0.9	0.4	0.6
Amaranthus polygamus (D)	0.0	0.0	0.0	0.	0.0	0.0	0.0	0.0

*Data not analysed statistically; $T_1 – T_4$: Tillage practices, $W_1 – W_3$: Weed management methods (for details see Materials and methods); A: 1st year maize; B: 1st year sunflower; C: 2nd year maize; D: 2nd year sunflower

after which its density gradually decreased due to crop rotation. The density of *Cyperus rotandus* remained high up to the 2nd year of maize, but thereafter its density was reduced in the subsequent sunflower crop. At the end of the study period the field was heavily infested with *Cynodon dactylon*, because zero tillage greatly favoured its growth. The conservation tillage system shifted the weed community composition towards problem weeds, e.g. grasses and vegetatively reproducing species (Zanin et al., 1997). In the present study it was also observed that *Cyperus rotundus* was smothered by *Cynodon dactylon. Amaranthus viridi* and *Amaranthus polygamus* were the dominant broad-leaved weeds observed during 1st year maize, but due to crop rotation, the density of *Amaranthus viridis* became negligible (0.1 m^{-2}) in 2nd year sunflower. Changes in agricultural practices and techniques, which alter conditions at the microhabitat level, might have influenced the composition of the weed flora.

Flaveria austerlagica (Table 1) appeared only in sunflower and its density declined from the 1st to the 2nd year of the cropping system. The appearance of *Gynandropsis pentaphylla* and *Portulaca quadrifida* was observed only in the 1st year and these weeds were completely eliminated in the 2nd year. The reverse trend was observed for *Aristolochia bracteata*, which did not appear in the 1st year of the cropping system, but appeared in the 2nd year cropping system.

The density of *Trianthema portulacastrum* was greater in maize than in sunflower, but due to crop rotation, the density gradually decreased during 2nd year maize. This result was in line with the findings of Teasdale et al. (2004). Similarly the density of *Datura metel* was higher during the initial period of study, after which its density was gradually reduced due to crop rotation (Stevenson et al., 1998).

These observations indicate that the cropping system contributed to the reduction in weed species composition. Crop rotation plays an important part in controlling the composition and density of weed flora, especially in conventional tillage systems.

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OPTIMUM HARVEST DATE OF MAIZE FOR BIOGAS AND SILAGE PURPOSES

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The dry matter and moisture contents of the aboveground vegetative organs and kernels of four maize hybrids were studied in Martonvásár at five harvest dates, with four replications per hybrid. The dry matter yield per hectare of the kernels and other plant organs were investigated in order to obtain data on the optimum date of harvest for the purposes of biogas and silage production.

It was found that the dry mass of the aboveground vegetative organs, both individually and in total, did not increase after silking. During the last third of the ripening period, however, a significant reduction in the dry matter content was sometimes observed as a function of the length of the vegetation period. The data suggest that, with the exception of extreme weather conditions or an extremely long vegetation period, the maximum dry matter yield could be expected to range from 22–42%, depending on the vegetation period of the variety. The harvest date should be chosen to give a kernel moisture content of above 35% for biogas production and below 35% for silage production. In this phenophase most varieties mature when the stalks are still green, so it is unlikely that transport costs can be reduced by waiting for the vegetative mass to dry.

Key words: maize, grain moisture, drying, biogas

Introduction

The cultivation of maize suitable for biogas production is similar to that of silage maize. In both cases the aim is to produce the maximum dry matter yield per hectare. This depends, among other things, on the length of the vegetation period, the plant height and the plant density. However, increases in these parameters are negatively correlated with the dry matter content of the kernels and other plant organs and positively with the water content of these plant organs (Hooper, 1925; Brown, 1962; Gunn and Christensen, 1965; Nevens et al., 1954; Rutger, 1969; Hadi, 1982; 1983; 2004; Hadi and Szundy, 1985; 1988; 1990; Berzsenyi and Lap, 2006a, b).

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As in the case of silage, the raw material required for biogas production will be used continuously and must therefore be suitable for storage without loss of quality. The key factor in deterioration-free storage is the dry matter content, the lower limit of which has been reported to be approx. 24% (Clark et al. 1973, White and Winter, 1979; Sheldrick, 1979; Hadi and Szundy, 1990). In general there is no upper limit. Only in the case of silage production is mention made of a slight deterioration in digestibility and in the nutrient, microelement and flavour contents as the dry matter content increases. Nevertheless, the correct choice of harvest date has a decisive effect on the dry matter yield, the dry matter distribution and the transportation costs, due to the higher or lower water content of the plant organs.

The present work was aimed at answering the following questions:

1. What changes are observed in the water content, dry matter percentage and dry matter yield of the kernels and other plant organs as a function of different harvest dates?

2. What range of harvest dates can be considered optimal when growing maize for silage or biogas production?

Materials and methods

The investigations were carried out on four hybrid maize varieties grown at a plant density of 60,000 plants/ha on chernozem soil with forest residues in Martonvásár with four replications. The hybrids included the silage varieties Bermasil (early) and Mv MSC 485 (mid-season) and the grain varieties Mv To 286 (early) and NK PX 9283 (mid-season).

Samples were taken from the whole aboveground part of five plants from each replication of each variety on five occasions (Sept. 11, 17, 24, Oct. 1, 8). The samples were divided at the nodes into the following parts: tassel, stalk above and below the ear, foliage above and below the ear, ear stalk, cob and kernels, which were separately labelled prior to drying in an oven at 95°C for 96 h in order to prevent the moist plant parts from suffering mass loss due to charring.

Thirty plant parameters were measured, counted or observed. The data were evaluated using analysis of variance.

Results

Values of morphological traits, averaged over the varieties, are presented in Table 1. It can be seen from the table that there was no significant change in the number of husks and kernel rows, in the plant and ear attachment height or in the number of leaves above and below the ear during the observation period. With the exception of plant height, these traits exhibited their maximum values during the differentiation phenophase, and later plant growth had little influence on them.

The maximum values of plant height were observed at silking (July 10–15), after which neither an increase nor a decrease was recorded, including during the sampling period.

Table 1
Plant parameters as a function of harvesting date

Parameters	Sampling date					$LSD_{5\%}$
	sep. 11.	sep. 17	sep. 24.	oct. 01.	oct. 08.	
No. of husks	9.9	9.5	9.8	10.4	9.8	0.6
Kernels/row	36.6	32.9	32.4	31.0	31.2	2.2
No. of kernel rows	14.8	14.7	14.8	14.6	14.7	0.5
Plant height (cm)	210.3	207.8	206.1	207.6	207.7	6.1
Ear attachment height (cm)	90.3	91.3	89.1	89.9	87.9	2.9
Ear length (cm)	21.7	19.2	18.9	18.4	17.8	1.0
Ear diameter (cm)	4.4	4.3	4.3	4.3	4.2	0.1
No. of leaves above the ear	5.8	5.6	5.6	5.8	5.6	0.5
No. of leaves below the ear	6.8	7.0	7.0	6.9	6.7	0.7

It was interesting to note a significant reduction in the ear length and the number of kernels per row. The former was probably due to drying after the completion of grain filling.

The growing period was dry during grain filling, resulting in a source deficit. The development of kernels at the ear tips came to a halt and the sugar solution previously transported to these kernels was redirected to kernels in the middle portion of the ear, which continued to grow, while those at the ear tips shrivelled up. In many cases the embryo completely disappeared, leaving only a small pericarp residue. This seems to be an example of the natural regulation within plants, which reduce their sink capacity when the source capacity declines.

The moisture content in various plant organs, averaged over the varieties, is presented in Table 2. With the exception of the tassel, the moisture content of the other plant organs was surprisingly high. This could be attributed to the fact that physiological maturity was observed in late September or early October. Until this stage is reached it remains possible for sugar solutions formed in green plant parts to reach the endosperm, even if the rate of transport slows. Active material transport is only possible in living plant tissues, the water content of which exhibits little fluctuation. The drying of the tassel probably began prior to the start of the observation period. This is understandable, as the tassel makes little contribution to the production and transport of sugar solutions. Due to self-shading, the leaves below the ear play a minor role in the production of sugar solutions, so part of their mineral content migrates into the upper leaves. Although the lower leaves have a high water content, they begin to dry earlier than the upper leaves. The drying rate of the husks was similar to that of the leaves below the ear, though this is not always the case. This phenomenon is widely known as stay-green, which means that the husks are dry, and the leaves are green.

Table 2
Changes in the water content (%) of various plant organs as a function of harvesting date

Parameters	Sampling date					$LSD_{5\%}$
	sep. 11.	sep. 17	sep. 24.	oct. 01.	oct. 08.	
Tassel	38.3	22.8	12.7	11.3	6.7	2.0
Leaves above the ear	71.3	69.4	65.8	61.8	44.7	1.8
Leaves below the ear	75.6	68.1	57.4	45.3	37.3	2.4
Husks	74.3	69.5	59.5	54.9	33.9	1.6
Stalk above the ear	77.6	78.3	77.6	77.5	75.3	1.4
Stalk below the ear	80.6	81.1	80.7	80.0	76.5	1.3
Ear stalk	83.8	83.6	83.3	84.0	81.8	0.8
Cob	63.9	64.5	63.6	62.1	58.8	1.4
Kernels	49.8	42.1	38.4	35.2	29.7	1.2
Whole plant	66.4	62.1	58.1	54.5	47.0	–

The ear stalk and the plant stalk above and below the ear are the moistest parts of the plant, and exhibited little change during the observation period. This, together with the fact that the cob had a high water content that only declined to a slight extent, confirms that there was no theoretical obstacle to material transport in the plants up to the end of September. This was also demonstrated by the retention of high moisture content in the kernels. Averaged over the varieties the grain moisture content was still around 30% on October 8.

It was interesting to note that there was an approx. 20% difference between the mean water content of the whole plant and the mean grain moisture content, averaged over the varieties. When the grain moisture content is known, this allows the mean moisture content of the whole crop to be estimated and the optimum harvest date to be pinpointed.

The dry yield per hectare of the various plant organs and the grain is presented in Table 3, averaged over the varieties. It can be seen that, with the exception of the grain, there was a significant drop in the dry matter yield per hectare for all the plant parts. This could probably be attributed partly to the loss of dry plant parts (e.g. tassel branches, leaf fragments) and partly to the source deficit, which caused the plants to export readily-soluble materials from the vegetative organs during the last third of the ripening period in order to ensure the development of the kernels. This is confirmed by the fact that there was a significant increase in the grain yield per hectare, averaged over the four varieties, between September 17 and October 1.

The dry matter yield per hectare of the whole plant did not change during the observation period. There was, however, an increase in the harvest index, indicating that, averaged over the varieties, the total dry matter yield per hectare had already formed prior to the start of the experiment. This means that when harvested for the purpose of biogas production, neither a further accumulation of dry matter, nor a reduction in drying costs can be achieved by delaying harvest, while in the case of silage production it is worth considering that the grain yield continued to increase during this period.

Table 3
Changes in the dry mass (q/ha) of the plant organs and in the harvest index as a function of harvesting date

Parameters	Sampling date					$LSD_{5\%}$
	sep. 11.	sep. 17	sep. 24.	oct. 01.	oct. 08.	
Tassel	28.23	24.39	23.83	21.47	20.67	1.8
Leaves above the ear	19.60	17.12	17.01	16.73	15.15	1.18
Leaves below the ear	22.31	21.97	21.23	19.65	18.61	1.37
Husks	9.97	7.57	7.28	6.95	6.83	0.82
Stalk above the ear	7.94	6.33	6.16	5.74	5.74	0.92
Stalk below the ear	31.89	27.37	26.27	23.8	24.12	2.37
Ear stalk	2.76	2.22	2.14	1.96	1.91	0.27
Cob	22.07	19.44	18.89	18.18	17.46	1.36
Kernels	93.01	98.91	105.33	110.37	113.86	10.27
Whole plant	237.78	225.32	228.14	224.85	224.35	14.25
Harvest index	39.11	43.89	46.17	49.09	50.75	3.85

Discussion

A comparison of the data in Tables 2 and 3 reveals that, averaged over the four varieties, the maximum dry matter yield had already formed prior to the start of the sampling period (Sept. 11). The mean water content of the whole plant exceeded 66%. A dry matter content of at least 24% (34% in the present case) is required if the raw material is to be stored without loss of quality for continuous use.

The mean grain moisture content at the first sampling was above 49%, but this did not mean that all the varieties had such high values. The grain moisture content of the very early varieties was around 40% or less, while that of the mid-season varieties was over 50%. By the following sampling date (Sept. 17) the mean grain moisture content had dropped to around 42%.

Both for the four varieties investigated and for other varieties examined in other experiments in other years, the maximum aboveground dry matter yield was obtained at grain moisture contents of around 42%. This can probably be attributed to the fact that the maximum dry matter yield of the aboveground plant organs coincides with silking, after which the kernels exhibit a rapid mass increase, the rate of which later slows. The total mass continues to increase to a certain point (up to a grain moisture content of 42%, or possibly later in favourable years). Losses in the mass of vegetative dry matter tend to be greater in source-deficient years and smaller when the weather is favourable for grain filling. This may equal or exceed the daily rate of increase in the grain yield per hectare. Except in years with very extreme weather conditions, the ideal harvest date (and the associated grain moisture content) is similar for biogas production and for silaging.

The data suggest that, depending on the year, the optimum harvest date is somewhere between the "half milk line" stage (42% grain moisture content) and physiological maturity (at present, 22–23% grain moisture content). Until a grain moisture content of 42% has been reached, the crop is not suitable for either silage or biogas production.

Maximum values of aboveground dry matter content and grain yield are not reached at the same time. For this reason, harvest can be recommended between a grain moisture content of 42% and 35% for biogas production and between 35% grain moisture content and physiological maturity for silaging. The latter can be justified by the fact that during this period the ratio of the grain to the whole plant biomass continues to grow. In average years the four varieties tested generally stay green up to a grain moisture content of 22%, thus confuting the argument that transport costs can be reduced by waiting for the high water content of the vegetative organs to dry.

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GENE EXPRESSION INVESTIGATIONS ON PLANT–PATHOGEN INTERACTIONS BETWEEN *Xanthomonas campestris* pv. *vesicatoria* AND PEPPER (*Capsicum annuum* L.) USING THE cDNA-AFLP TECHNOLOGY

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In order to target factors involved in plant–pathogen interactions, gene expression differences were investigated on pepper (*Capsicum annuum* L.) plants after artificial infection with the bacterial pathogen *Xanthomonas campestris* pv. *vesicatoria.* Amplified Fragment Length Polymorphism investigations on reverse transcribed DNA fragments (cDNA-AFLP) were used to compare the expression profiles of parental lines and of resistant and susceptible individuals from pepper populations segregating for the *gds* gene, which confers a general defence system in pepper. In total, 73 transcript-derived fragments (TDFs) displaying differential expression patterns could be identified (presence-absence and/or different time courses in resistant and susceptible genotypes). Of these, 67 fragments were cloned and sequenced. In the case of several TDFs, sequence comparisons revealed close homologies to genes known to be responsible for abiotic stress or biotic elicitors, presenting potentially interesting targets for more detailed studies on gene expression and signal transduction.

Key words: cDNA-AFLP, pepper, plant–pathogen interactions, general defence system

Introduction

Foliage and fruit spot caused by the Gram-negative plant pathogen *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) is one of the most important bacterial diseases of tomato and pepper. Outbreaks of bacterial spot epidemic usually occur during warm, humid weather conditions and can result in significant crop losses. The bacterium survives in seeds, in infected crop debris and in weeds. Leaves are infected through the stomata and the fruits through wounds. It is spread from one plant to another by wind, rain-splash, overhead irrigation or plant-to-plant contact. Disease severity depends on moisture and temperature and the level of resistance within the cultivar. So far, more than 140 pathovars have been defined within *X. campestris*. The pathovar "*vesicatoria*", which was originally described as a homogeneous group causing one consistent disease, appears on the basis of DNA hybridization experiments to be composed

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of two completely unrelated genomic types, which now constitute *X. vesicatoria* and a subgroup of *X. axonopodis* (Vauterin and Swings, 1997). More recently, in an extensive revision of the genus *Xanthomonas*, the name *Xanthomonas vesicatoria* (ex Doidge) was proposed (Vauterin et al., 1995). On the basis of virulence, three groups of *X. vesicatoria* have been distinguished till now: the tomato group (*XcvT*) is virulent only on tomato, the pepper group (*XcvP*) only on pepper, and the pepper/tomato group (*XcvPT*) on both pepper and tomato (Reifschneider et al., 1985). Within the *XcvPT* and *XcvP* groups, pathogen races (1, 2 and 3) can be distinguished by their ability to cause disease on various pepper lines.

Resistance in pepper to *X. vesicatoria* is generally associated with the hypersensitive reaction (HR). To date, three different *Xanthomonas* resistance genes have been described in pepper, which are localized on the *Bs1*, *Bs2* and *Bs3* loci (Hibberd et al., 1987). The genes *Bs1* and *Bs*3 confer HR-based resistance to race 2 and race 1, respectively, while the *Bs2* gene product restricts races 1, 2 and 3. These dominant alleles show independent monogenic Mendelian segregation. The corresponding avirulence genes (*avrBs1*, *avrBs2* and *avrBs3*) have been cloned from *Xcv* and shown to be essential for controlling resistance (Minsavage et al., 1990). By contrast, a new type of non-hypersensitive *Xanthomonas* resistance, called *gds*, was recently found in the pepper genotype PI 163192, which is effective against both *Xcv* and other pepper pathogens. This resistance is based on the recessive *gds* (*general defence system*) gene. Unlike the hypersensitive reaction, in the case of the *general defence reaction* the cells attacked by the pathogens do not become necrotic, as the *gds* gene confers a previously unknown reaction manifested in cell enlargement and cell division, resulting in the thickening and slight chlorosis of the infected tissues. This type of plant reaction, which strengthens the defence of cells affected by the stress, has a low stimulus threshold and an exceptionally high reaction speed, and is not specific to the pathogen. The general defence system is thought to be a fundamental plant character, which may also provide protection against abiotic and biotic stress factors (Szarka and Csilléry, 2001a, b; Szarka et al., 2002; 2006).

Several pathogen-responsive genes involved in the pepper–*Xanthomanas* relationship have been identified recently and their expression has been studied using various expression profiling techniques such as cDNA subtractive hybridisation and microarrays (Jung and Hwang, 2000; Lee and Hwang, 2003; Jung et al., 2005; Lee et al., 2004).

Since the introduction of the Amplified Fragment Length Polymorphism (AFLP) technique (Vos and Hogers, 1995) several modifications have been made in the original protocol. Expression profiling based on the AFLP fingerprinting of reverse-transcribed mRNA fragments (cDNA-AFLP) (Bachem et al., 1996; 1998) is commonly used to display the transcriptome of a specific tissue or developmental stage. In this study, a strategy based on cDNA-AFLP was combined with Bulked Segregant Analysis (BSA) to monitor pathogenesis-related gene expression changes in pepper.

Materials and methods

Plant material

All the plant material used in these studies originated from the breeding material of Budakert Ltd. Plants from the F_2 to F_4 populations, representing crosses between donor genotypes containing the *gds* gene and susceptible recipient lines, were grown in growth chambers. At least 3 resistant and 3 susceptible plants from each segregating population and 6 to 8 plants of each parental genotype were chosen for the experiments. Three leaves on each selected plant were infiltrated with a fresh bacterial culture of *Xanthomonas campestris* pv. *vesicatoria* (10^8 cells/ml). The infected leaves were harvested at different times after inoculation (0, 2, 12, 24 h), immediately frozen in liquid nitrogen and stored at –70°C until processing. At each harvesting time three infected leaves, each taken from different plants and canopy levels, were chosen and pooled to make bulks for RNA isolation.

cDNA-AFLP

Frozen pepper leaf material (0.5 to 1 g) was ground with a mortar and pestle. Total RNA was isolated by LiCl precipitation following phenol/chloroform extraction. PolyA+ RNA fractions were recovered using paramagnetic particles conjugated to oligo-dT (Dynal, Oslo, Norway), and subsequently served as primer for the cDNA synthesis using AMV reverse transcriptase. During the following steps, cDNA-AFLP analysis was performed according to Bachem et al. (1996; 1998). Primary cDNA templates were simultaneously digested with the restriction enzymes *TaqI* (frequent cutter) and *AseI* (rare cutter), and the following double-stranded adapters were ligated to the cohesive ends:

TaqI adapter:

```
5' CTCGTAGACTGCGTACC 3'
3'        CTGACGCATGGAT 5'
```

AseI adapter:

```
5' GACGATGAGTCCTGAC 3'
3'      TACTCAGGACTGGC 5'
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During the pre-amplification reactions, adapter-ligated cDNA fragments were amplified, using adapter-specific primers having a single selective nucleotide overhang (*Taq*+1A, *Ase*+1C). Typical PCR parameters were: initial denaturation at 94°C for 3 min followed by 35 cycles at 92°C for 1 min, 56 °C for 1 min and 72°C for 1 min and terminated by a final extension at 72°C for 7 min. The pre-amplification reactions were diluted 10-fold and used as template for the second amplification step under the following conditions: 11 cycles with an initial annealing temperature of 65°C, decreasing 0.7°C in each cycle (94°C for 30 s, 65 to 57.3°C for 30 s, 72°C for 1 min) and subsequently 23 cycles at an annealing temperature of 56°C (94°C for 30 s, 56°C for 30 s, 72°C for 1 s). Adapter-specific primers with two or three nucleotide overhangs were used for the selective AFLP amplifications. The *AseI* primers were end-labelled by phosphorylation using [γ-^{33}P]ATP and T4 polynucleotide kinase. The amplification products were separated on 8% polyacrylamide gels and visualised by autoradiography.

Analysis of TDFs

Bands of interest were excised from the dried gels and reamplified using the primers used in the pre-amplification reactions. The PCR products were cloned in pGEM5zf+T vector (Promega) and the plasmid clones were sequenced on an ABI 3100 (Applied Biosystems) sequencer using standard sequencing procedures. Database searches using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) were performed at the NCBI World Wide Web server network service. Additionally, for all the corresponding sequences, BLAST analyses were performed on a local sequence database containing about 30,000 public pepper sequences.

Results

In order to adapt and optimize the cDNA-AFLP technique, 48 primer–enzyme combinations were tested. The most critical parameters of amplification proved to be the overhanging selective ends of the *Ase* primers. In general, the fragment patterns were too complex for primers with one nucleotide overhang, and poor for primers with three nucleotide overhangs. Primers with two-nucleotide extension proved to be optimal and were consistently used thereafter. Great differences were found in the nucleotide composition of the overhangs: *Ase* primers bearing CG, CC and CT overhangs provided optimal fragment complexity, while *Ase* primers with AA, AT and CA extensions provided non-specific or weak amplification products. Seventy-three fragments showing differential expression patterns between resistant and sensitive genotypes or at different times were isolated from the polyacrylamide gels (Fig. 1). The isolated fragments were re-amplified and cloned into pGEM5zf+T-vector. Cloning was successful in the case of 67 fragments. All these clones were sequenced for further comparison and characterisation.

Each sequence was inspected and edited manually. The adapter sequences were removed from the ends. Transcript-derived fragments (TDFs), ranging from 22 to 223 bp in size, were used as queries for BLAST analyses. No function could be assigned to 26 fragments (39%). Forty fragments showing significant homologies to existing database entries are listed in Table 1 and the functional grouping of the most relevant homologues is summarized in Table 2.

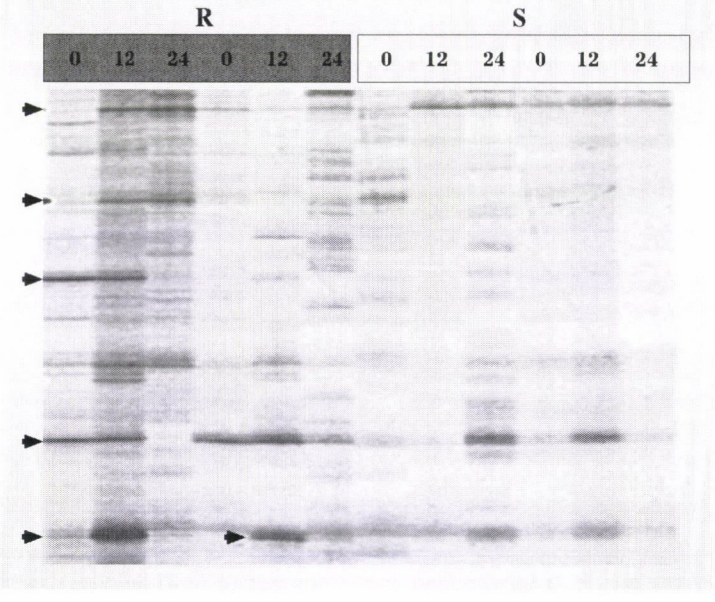

Fig. 1. cDNA-AFLP fragments differently expressed in *Xanthomonas* resistant (R) and sensitive (S) genotypes following artificial infection. Fragments indicated with arrows were cut out of the gel, cloned and sequenced. Leaf samples were taken immediately (0), 12 and 24 hours after infection

Table 1

Differently expressed *Capsicum annuum* cDNA fragments showing significant homology to existing database entries. Clones were categorised on the basis of their expression pattern at different time intervals following *Xanthomonas* infection

No.	Code	Expression pattern S A	S B	R A	R B	L., bp	Most significant homologues	Accesion No.	Score	E-value
1	*XCE-03*	+				59	*Oryza sativa* cultivar Nipponbare clone, hypothetical protein	AC079935	40.1	0.046
2	*XCE-09*	+				115	*Lycopersicon esculentum* cDNA clone, *Pseudomonas*-resistant tomato	AI773310	58	5e-7
3	*XCE-10*		+			223	*Cabomba caroliniana* 26S ribosomal RNA gene	AF479239	281	3e-74
4	*XCE-11*	+				11	*Solanum tuberosum* cDNA clone STMJC50 (mixed potato tissues)	BQ517244	159	2e -37
5	*XCE-17*	+				88	*Capsicum annuum* cDNA sequence KS0801A10 KS08. relation to HR	BM068179	101	2e-20
6	*XCE-19B*	+				89	*Licopersicon esculentum* cDNA clone cTOE4C7 (tomato crown gall)	BG131407	92	2e-17
7	*XCE-020*		+			68	*Ipomoea* nil cDNA clone:jm22k12	BJ573046	36.2	0.87
8	*XCE-21*	+				31	*Mesembryanthemum crystallinum* cDNA clone NaCl treatment	CA840428	34.2	0.97
9	*XCE-22*	+				68	*Arabidopsis thaliana* disease resistance protein-like	AB005248	36.2	0.34
10	*XCE-23*	+				158	*Licopersicon esculentum* cDNA clone cLES10J14, susceptible	AI780173	44	0.010
11	*XCE-28*			+		88	*Capsicum annuum* KS08018A10 KS08 cDNA	BM068179	174	2e-42
12	*XCE-29*			+		83	*Capsicum annuum* KS01041A10 KS01 cDNA relation to HR	BM062305	167	5e-40
13	*XCE-30*			+		199	*Capsicum chinense* KS04182T70 KS04 cDNA relation to HR	CB185098	133	2e-29
14	*XCE-31*		+			91	*Beta vulgaris* cDNA clone 024-027-F10 (developing root)	BQ593091	36.2	1.3
15	*XCE-33*		+			79	*Capsicum annuum*cDNA KS10026H12 KS10 relation to HR	CA519532	155	2e-36
16	*XCE-34*		+			117	*Capsicum annuum*cDNA KS12065B02 KS12 relation to HR	CA526054	232	1e-59
17	*XCE-35*		+			79	*Capsicum annuum*cDNA KS10026H12 KS10 relation to HR	CA519532	155	2e-36
18	*XCE-36*		+			46	*Gossypium arboreum* fibre cDNA GA__Eb0040L01f	BG447126	36.2	0.49
19	*XCE-39*			+		90	*Oryza sativa* Nipponbare clone OSJNBa0084C09	AC016781	38.2	0.32
20	*XCE-40*			+		139	Tobacco E 22 gene for thaumatin	NTE22TLP	351,4	8e-13
21	*XCE-45*	+				128	*M. truncatula* cDNA NF081H09ST developing stem	AW694961	48.1	5e-04
22	*XCE-46*		+			173	*Licopersicon esculentum* cDNA cLET43P5 (tomato mixed elicitor)	AW443286	44.1	0.011
23	*XCE-47*	+				50	*Capsicum annuum*cDNA KS07007H07 KS07 relation to HR	BM065830	89.7	4e-17
24	*XCE-51*		+			113	*Arabidopsis thaliana* sequence flanking Ds3 end of Ds-Trap insertion line	AY202677	40.1	0.11
25	*XCE-53*		+			79	*Capsicum annuum*cDNA KS10026H12 KS10 relation to HR	CA519532	155	2e-36
26	*XCE-54*	+				193	*Licopersicon esculentum* cDNA cTOS21G19 suspension culture	BI209725	149	3e-34
27	*XCE-56*	+				22	*Capsicum annuum*cDNA KS01017B02 KS01 relation to HR	BM060662	44.1	4e-04
28	*XCE-57*	+				98	*Licopersicon esculentum* EST clone fle73rep petal and stamen	AJ319982	81.8	3e-14
							*Capsicum annuum*cDNA KS01042D04 KS01	BM062408	81.8	3e-14

Table 1 continued

No.	Code	Expression pattern S A	S B	R A	R B	L., bp	Most significant homologues	Accesion No.	Score	E-value
29	*XCE-58*	+				94	*Capsicum annuum*cDNA KS01042D04 KS01 relation to HR	BM062408	58.0	4e-07
30	*XCE-61*	+				84	*Licopersicon esculentum* cDNA cC-esflcLEL17N05a1 flower development	BG627505	42.1	0.019
31	*XCE-62*	+				65	*Arabidopsis thaliana* chromosome 1 centromere region, BAC clone:T23P23.	AB086245	38.2	0.21
32	*XCE-63*	+				49	*Arabidopsis thaliana* chromosome 2 clone F7B1	AC006586	38.2	0.14
33	*XCE-64*			+		60	*Pinus taeda* cDNA clone NXRV117_D01 root maturation	BQ698938	38.2	0.19
							*Capsicum annuum*cDNA KS11035D03 KS11 relation to HR	CA521847	36.2	0.73
34	*XCE-66*				+	193	*Licopersicon. esculentum* cDNA cTOS21G19 suspension culture	BI209725	129	2e-28
35	*XCE-67*			+		139	*Capsicum annuum*cDNA KS07006C01 KS07 relation to HR	BM065678	274 b	5e-72
36	*XCE-68*			+		109	*Solanum tuberosum* cDNA STMGO80 (mixed tissues)	BQ507172	87.7	5e-16
37	*XCE-69*			+	+	82	*Lactuca sativa* cDNA clone QGB18D17	BQ852512	34.2	4.4
38	*XCE-70*				+	68	*Medicago truncatula* cDNA MtBC22E05R1	AL384498	34.2	3.5
							Glomus intraradices mycorrhiza			
39	*XCE-71*				+	64	*Ipomoea* nil cDNA clone:jm22k12 flower and bud tissue	BJ573046	36.2	0.80
40	*XCE-72*			+		46	*Arabidopsis thaliana* chromosome 3, P1 clone:MKA23	AP001306	40.1	0.031

A: early; B: late; L: length

Table 2
Functional grouping of the isolated transcripts with relevant known homologues

	Biotic stress-induced genes:	Abiotic stress (salt, cold)-induced genes:	Organogenesis-related genes
Up-regulated in sensitive genotypes	*XCE-11, XCE-17, XCE-19, XCE-61*	*XCE-57, XCE-58*	–
Up-regulated in resistant genotypes	*XCE-29, XCE 40, XCE-46, XCE-64, XCE-67, XCE-68*	*XCE-39, XCE-40, XCE-53, XCE-64, XCE-66, XCE-72*	–
–	–	–	*XCE-57, XCE-59, XCE-61, XCE-66*

About 50% of the TDFs (19 out of 40) showed up-regulation in sensitive genotypes. The induction of 8 TDFs was triggered immediately after infection, while 11 fragments appeared relatively late (12–24 hours after inoculation). The remaining 21 TDFs were expressed exclusively or showed up-regulation in resistant genotypes. In this group, a relative quick and strong post-infection expression could be detected for 13 TDFs, decreasing over time, except *XCE-69*, which persisted still at 24h. 8 TDFs displayed late (>12 hours after infection) up-regulation.

Discussion

Bulked segregant analysis (BSA) (Michelmore et al., 1991) is an effective tool for the targeting of loci that are responsible for the manifestation of a monogenic character in a segregating population. In the present investigations resistant and susceptible plants/samples were grouped and the gene expression profiles of each pool were compared under non-induced and pathogen-induced conditions in different time courses. This strategy potentially facilitates the identification of transcripts that are exclusively present in one type of pool and are absent in others, as well as the identification of transcripts that are induced or repressed after pathogen inoculation.

DNA microarray-based techniques have become popular tools for genome-wide transcriptome analysis. However, because of their high technical demand, microarrays are only available to a minority of laboratories. In addition, microarrays are only feasible when extensive sequence information and cDNA libraries are available. For this reason cDNA-AFLP was chosen as the transcript profiling technique. In comparison to other gel-based expression-profiling techniques, such as mRNA differential display (DDRT-PC) (Liang and Pardee, 1992), cDNA-AFLP has better reproducibility, because stringent PCR amplification conditions are applied in all steps. One drawback of the AFLP technique is that only DNA fragments with appropriate sequence patterns can be targeted. However, this can be circumvented by using a wide variety of different restriction enzymes and primers. The cDNA-AFLP technique has been successfully applied for the display and isolation of differentially expressed genes in plant development (Goupil et al., 2003), stress response (Simoes-Araujo et al., 2003) and plant–pathogen interactions (Durrant et al., 2000; Van der Biezen et al., 2000; Eckey et al., 2004).

In this study 73 differentially expressed fragments were identified and cloned, of which 14 cDNA clones were confirmed to have high sequence similarity to defence- or pathogenesis-related genes. One particularly interesting clone is *XCE-40*, which seems to be strongly expressed in resistant genotypes 24 hours after inoculation, while little or no expression could be detected in the same stage in sensitive genotypes. It shows great similarity to a tobacco thaumatin gene expressed in leaves infected with Tobacco Mosaic Virus. Thaumatin-like proteins are generally considered to be pathogenesis-related (PR) proteins in plants (for reviews see Breiteneder, 2004; Linthorst, 1991). Genes for pathogen-induced thaumatin-like proteins were identified in wheat (Rebmann et al., 1991), rice (Reimmann and Dudler, 1993) and tomato (Rodrigo et al., 1993). Furthermore, thaumatin-like proteins are also involved in osmotic regulation and drought tolerance (Jung et al., 2005) and abiotic stress (Zhu et al., 1993). In pepper the isolation and characterisation of a gene for thaumatin-like protein (PepTLP) was reported recently, which showed higher expression in anthracnose-inoculated pepper fruits in incompatible interactions than in compatible ones. Furthermore, PepTLP gene expression proved to be stimulated by jasmonic acid and wounding (Kim et al., 2002).

Several TDFs represent identical parts of public pepper EST sequences that were isolated from a cDNA library related to hypersensitive response against pathogens, e.g. the clone *XCE-17*, which is probably a homologous part of the pepper EST sequence BM068179. In the present experiments this clone was expressed in both sensitive and resistant genotypes. Another potentially interesting transcript is *XCE-22*, which shows homology to a disease resistance-like gene from *Arabidopsis thaliana* (accession ID: AB005248).

In conclusion, these preliminary results show that a combination of cDNA-AFLP and Bulked Segregant Analysis could be an effective tool for studying pathogenesis-related changes in expression. More detailed genomic and gene expression analysis will be required to verify the involvement of the identified transcripts in pathogenesis-related processes.

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INDUCTION OF PARTHENOCARPY IN WATERMELON (*Citrullus lanatus*) CULTIVARS BY GAMMA IRRADIATION

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It was found that diploid seedless watermelon can be produced by pollination with partially functional pollen which was irradiated with gamma rays at doses of 600 and 800 Gray (Gy). The diploid seedless fruit was very similar to normal fruit in days to maturity from pollination and rate of fruit set. The number of empty seeds in the diploid seedless fruit varied for the cultivars used in this study. No correlation was found between the number of empty seeds in seedless fruit and the number of normal seeds in normal fruit. Also, the results indicated that seedless watermelon cultivars have a significant increase in total sugar and carotenoids (lycopene and β-carotene) content, providing an important source of phytonutrients in the diet. The pollen tube of pollen irradiated with gamma radiation penetrated normally into the synergid and sperm cells were discharged. Subsequently, the egg nucleus and sperm nucleus became attached to each other in the egg cell and a globular embryo was formed. However, the embryo failed to differentiate into organ tissues and degenerated. It was suggested that seedless fruit induced by gamma rays had a beneficial effect in increasing the quantity and quality of watermelon yield via increases in the carotenoid, total sugar content and fruit weight. Also, in some cultivars there was a tendency for the thickness of the rind to decrease.

Key words: *Citrullus lanatus*, embryo, parthenocarpy, pollination, carotenoids, gamma irradiation

Introduction

The watermelon (*Citrullus lanatus*) belongs to the *Cucurbitaceae* (or gourd) family and is the largest edible fruit. Originating in Africa (Wein, 1997), watermelons were first cultivated 5,000 years ago in Egypt, where testaments to their legacy were recorded in hieroglyphics painted on the walls of buildings. The fruit was held is such regard that it was placed in the tombs of many Egyptian kings. When ripe, the sweet juicy pulp is eaten fresh, and the rind is sometimes preserved (Dupree et al., 1953). The seeds are roasted as a snack or

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ground into an ingredient in oils or sauces. An edible syrup or fermented beverage can be made from the juice (Webster and Romshe, 1951). Watermelon juice may increase blood concentrations of carotenoids (lycopene and β-carotene). Carotenoids are important to humans because they have antioxidant activity and prevent free radicals from causing harm to the body, and have protective effects against heart disease and certain cancers, such as prostate, bladder, breast, cervical, endometrial, lung and colorectal (Edwards et al., 2003). Watermelon is an excellent source of vitamin C and a very good source of vitamin A, notably through its concentration of β-carotene (Olson, 1999).

Pollen irradiation is the most widely used technique to induce *in situ* haploid plants (Musial and Przywara, 1998). This technique has been applied to several crops (e.g. apple, cucumber, kiwifruit, pear, rose, watermelon). Although pollen irradiation has been used for many years, Blakeslee et al. (1922) were probably the first who used it. Pollination with irradiated pollen was carried out by Nishiyama and Uematsu (1967) in *Lycopersicon*, Le Deunff and Sauton (1994) in cucumber, and Musial and Przywara (1998) in kiwi fruit. Chalak and Legave (1997) showed that parthenogenesis induced by heavily gamma-irradiated pollen can produce haploid and maternal diploid (in fact trihaploid and hexaploid, respectively) plants in *Actinidia deliciosa*. However, an alternative irradiation technique using soft X-ray irradiation of the pollen is used for the production of seedless watermelon (Sugiyama and Morishita, 2000). In the case of gamma-rays it is thought that pollen activity becomes lower rapidly after gamma-irradiation, suggesting that the energy of gamma-rays is too strong. There are no disadvantages to soft X rays compared with gamma-irradiated pollen (Sari et al., 1994). The gamma-irradiated watermelon pollen technology used for producing haploid and dihaploid watermelon plants is the subject of a US Patent (Anonymous, 2008), which demonstrates that the technique described in the present work is suitable for other practical purposes as well.

Seedless watermelon fruits are popular with consumers because they are very easy to eat. At present, seedless watermelons are produced by utilizing triploid plants. This method was developed about 50 years ago (Terada and Masuda, 1943; Kihara and Nishiyama, 1947; Kihara, 1951). Seedless watermelon fruits have also been produced using plant growth regulators (Wong, 1938; Terada and Masuda, 1940; 1941; Hayata et al., 1995). Hybrid seedless (triploid) watermelons have been grown for over 40 years in the United States. Since 1990, triploid or 'seedless' watermelons have been in great demand (Sanford, 1990; Beste et al., 1998; Mussen, 1999). Watermelon pollen is not windblown; the flowers are almost exclusively insect pollinated (Porter, 1933). A 'seedless' watermelon actually has seeds, but they are small, translucent white seed coats that are eaten along with the fruit and are not even noticed (Beste et al., 1998). In botany and horticulture, parthenocarpy (literally meaning virgin fruit) is the natural or artificially induced production of fruit without fertilization of the ovules. (The biological term parthenogenesis refers to the development of

an egg without fertilization). The fruit is therefore seedless. Some parthenocarpic cultivars have been developed as genetically modified organisms (Pandolfini et al., 2002). Seedless cultivars are produced by crossing a tetraploid (4×=44) inbred line as the female parent with a diploid (2×=22) inbred line as the male parent of the hybrid (Beste et al., 1998). The reciprocal cross (diploid female parent) does not produce seeds. The hybrid is a triploid (3×=33), and is female and male sterile. Triploid plants have three sets of chromosomes, and three sets cannot be divided evenly when they enter two daughter cells during meiosis (the cell division process that produces the gametes). Since the triploid hybrid is female sterile, the fruit are seedless. Because the triploid is also male sterile, it is necessary to plant a diploid cultivar in the production field to provide the pollen that stimulates fruit to form. Usually, one-third of the plants in the field are diploid and two thirds are triploid. The development of triploid cultivars adds several problems to the process of watermelon breeding: reproduction from hybrid seeds (making them very expensive); extra time required for the development of tetraploids; additional selection against sterility and fruit abnormalities; choice of parents for reduced seed coat production; a reduction in the seed yield per hectare obtained by seed companies; reduced seed vigour for the grower; and the necessity for a diploid pollenizer, taking up one-third of the grower's production field. One problem affecting triploid hybrids is the empty seed coats (coloured or white) in the fruit. Under some environmental conditions, fruit are produced with large obvious seed coats that are objectionable to consumers.

Although the cost of producing triploid seedless watermelon seed is very high, plant growth regulators cannot be used from a safety point of view. Because the current production methods for seedless watermelon have various shortcomings (Kihara, 1951; 1958; Yamamuro, 1978), it is necessary to develop a new method to produce seedless watermelon fruits which would be more convenient for and acceptable to farmers. A more practical method than the current methods has been successfully developed, involving only pollination with pollen irradiated by gamma rays. These seedless watermelons can be grown using ordinary cultivation practices. In this paper, the method for producing seedless watermelons will be discussed in detail.

Materials and methods

Plant material and growth conditions

Watermelon cultivars (Giza 1, Giza 21, Crimson Sweet, Sugar Baby, Sun Gold, Korgan, Sharmen and Jingxin) were obtained from the National Agriculture Research Center, Giza, Egypt. The experimental field studies were conducted at the Barrage Horticultural Research Station, Kalubia Governate. Two field experiments were conducted during the growing seasons of 2007 and 2008. Each of the tests was established with crops planted in single rows on raised beds. The soil type in the plot area is clay loam. The experiment was arranged in a randomized block design with three replications. Each experimental plot took up an area of 24.0 m^2 (6 × 4 m) and included 20 plants with 1.2 × 1.0 m spacing. There was a gap 3 m wide between the plots. The melons were furrow-irrigated throughout the season.

Experiment I. Effect of irradiation dose on number of seeds

Watermelon cultivars Giza 1, Giza 21 and Crimson Sweet were sown on April 2, 2007. Male flowers were harvested in the morning when they bloomed. The prepared male flowers were irradiated with different doses of gamma (^{60}Co) irradiation, 0 (control), 75, 150, 300, 600 and 800 Gray (Gy) at the Middle Eastern Regional Radioisotope Center for the Arab Countries (Dokki, Cairo, Egypt) with a strength of 500 Ci and a dose rate of 0.54 Gy/min. Female flowers were bagged at the bud stage to avoid uncontrolled pollination. The flowers were hand-pollinated by brushing the stigmas with irradiated pollen and re-bagged after pollination to eliminate contamination by foreign pollen. The bags remained in place until the styles withered (approx. 1 week after pollination). Control pollinations with non-irradiated pollen were performed at the same time. Ten plants per cultivar were included in the experiments. Mature fruits were harvested at about 54 days after pollination for Giza 1, 56 days for Giza 21 and 54 days for Crimson Sweet and the number of normal and empty seeds was counted.

Experiment II. Effect of irradiation on fruit set

Giza 1, Giza 21 and Crimson Sweet were sown on March 25, 2007. Male flowers were irradiated with 600 Gy on the blooming day. After irradiation, the pollen was used for investigations of the rate of fruit set.

Experiment III. Effect of irradiation on growth and chemical characteristics

Eight cultivars (Giza 1, Giza 21, Crimson Sweet, Sugar Baby, Sun Gold, Korgan, Sharmen and Jingxin) were sown on March 20, 2007. Six plants per cultivar were used in the treatments. Blooming male flowers of Giza 1 were irradiated with 600 Gy of gamma rays and immediately used to pollinate each watermelon plant. Unirradiated pollen of Giza 1 was used as the control. After harvest, fruit weight, flesh colour, rind thickness and days to maturity were recorded. The total sugar content in the watermelon juice was determined as described by Davis et al. (2008) using an Enzymatic Bioanalysis kit (Boehringer Mannheim/R-Biopharm AG, Germany). The carotenoid (lycopene and β-carotene) contents in the fruit were measured according to Perkins Veazie et al. (2004) and Davis et al. (2007).

The mean differences between the characteristics of normal and seedless fruit were determined by the t-test.

Experiment IV. Effect of irradiation on embryo abortion

Giza 1 was sown on January 2, 2008 and male flowers were collected in the morning at anthesis. The blooming male flowers were irradiated with a 600 Gy dose of gamma rays and were immediately used for pollination. A minimum of 7 fruits were selected at random at 0 to 30 days after pollination with irradiated or unirradiated pollen and rapidly fixed with a formalin-acetic acid alcohol (FAA) solution. The fixed specimens were dehydrated in a graded t-butanol series, embedded in paraffin and sliced into 10 µm-thick serial sections. The sections were stained with haematoxylin and safranin, or with haematoxylin, safranin and fast green. Coverslips were put on the stained sections using balsam prior to microscopic examination (Boeke and Ortiz-Crespo, 1978; Neinhuis and Edelmann, 1996).

Results

Experiment I

The number of normal seeds in Giza 1, Giza 21 and Crimson Sweet was significantly reduced when female flowers were pollinated with irradiated pollen (Table 1). In all three cultivars no normal seeds, only empty seeds were observed at 600–800 Gy (Fig. 1). Giza 1 had more empty seeds than Giza 21 and Crimson Sweet. At an irradiation dose of 600 Gy the number of empty seeds in Giza 1

was about 400, with 376 in Giza 21 and 200 in Crimson Sweet, while at a dose of 800 Gy, this figure declined to about 293 in Giza 1, 282 in Giza 21 and 175 in Crimson Sweet. With increasing gamma irradiation doses there was thus a tendency for the total number of seeds in the three cultivars tested (Giza 1, Giza 21 and Crimson Sweet) as compared with the control samples.

Experiment II

No differences in fruit set were detected between pollen irradiated with gamma rays (600 Gy) and unirradiated pollen (control) in Giza 1, Giza 21 and Crimson Sweet (Table 2).

Table 1
Effect of different doses of gamma irradiation on the number of seeds in watermelon fruit

Cultivars	γ-irradiation dose (Gy)	No. of normal seeds	No. of empty seeds			Total No. of seeds
			Total	White	Coloured	
Giza 1	0.0	608±55	35±4	35	0	643±39
	75	130±5	470±56	452	18	600±66
	150	63±7	440±60	420	20	503±91
	300	10±1	462±101	449	13	472±62
	600	0±0	400±63	395	5	400±63
	800	0±0	293±45	286	7	293±45
Giza 21	0.0	502±60	71±12	70	1	573±53
	75	216±31	279±17	257	22	495±100
	150	102±7	306±24	291	15	408±83
	300	23±2	404±13	372	32	427±53
	600	0±0	376±48	375	1	376±48
	800	0±0	282±50	282	0	282±50
Crimson Sweet	0.0	424±39	43±5	43	0	467±61
	75	114±10	281±40	272	9	395±16
	150	70±13	239±70	226	13	309±47
	300	11±1	255±15	250	5	266±29
	600	0±0	200±21	198	2	200±21
	800	0±0	175±8	171	4	175±8

Values are mean ± SE.

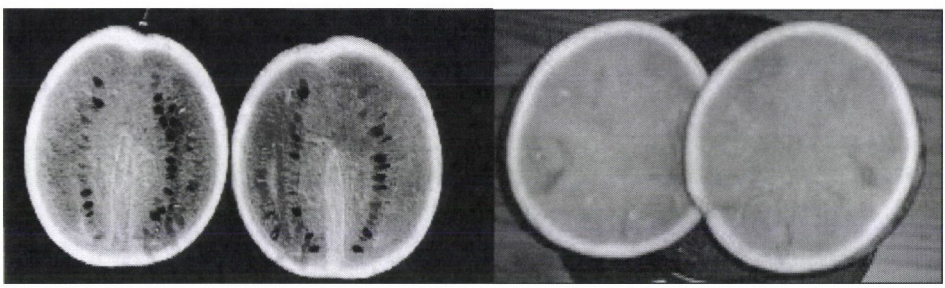

Fig. 1. Diploid seedless watermelon (Giza 1) cultivar produced using pollen irradiated with gamma rays (600 Gy). Left: Normal fruit. Right: Seedless fruit

Table 2
Effects of irradiated pollen on the fruit set of watermelon

Cultivars	γ-irradiation dose (Gy)	No. of pollinated female flowers	No. of fruit set	Fruit set (%)
Giza 1	0.0	62	36	58.1
	600	67	40	59.7
Giza 21	0.0	60	33	55.0
	600	56	30	53.6
Crimson Sweet	0.0	57	38	66.7
	600	53	36	67.9

Experiment III

The weight of the seedless fruit of Korgan was similar to that of the normal fruit, but in Giza 1, Giza 21, Crimson Sweet, Sugar Baby, Sun Gold and Jingxin the fruit weight increased significantly by 11%, 16%, 12%, 10%, 11% and 6%, respectively. Sharmen, however, exhibited the reverse tendency, as the fruit weight decreased significantly by 18% compared with the control (Table 3). Seedless fruit of Giza 21 and Korgan had a deeper flesh colour than the control. The rind of Korgan and Sharmen was thinner in seedless fruit than in normal fruit, but in Giza 21 and Sun Gold the tendency was reversed (Table 3). The days to maturity of seedless fruit was similar to that of normal fruit, so there was no consistent relationship between these fruit characteristics and irradiation with gamma rays (600 Gy).

In the seedless fruit, only empty seeds were found, but their frequency ranged widely. Sun Gold showed the largest number of empty seeds (221). While normal fruit of Giza 21 contained 491 seeds, the seedless fruit had only 15 empty seeds (Table 3). The seedless fruit of Giza 1, Giza 21, Sugar Baby, Jingxin, Crimson Sweet and Sharmen had 10, 15, 25, 61, 63 and 76 empty seeds, respectively. The seedless fruit of Giza 1, Giza 21, Sugar Baby, Sun Gold, Korgan, Jingxin and Sharmen had significantly higher sugar content than the normal fruit (20%, 10%, 11%, 10%, 8%, 19% and 9%, respectively), while there was no difference for Crimson Sweet (Table 4). The seedless fruit of Giza 1, Giza 21, Sugar Baby, Sharmen and Jingxin had a significantly higher level of lycopene than the normal fruit (23%, 12%, 14%, 21% and 20%, respectively) while the seedless fruit of Crimson Sweet and Korgan did not differ from normal fruit in this respect. Seedless fruit of Sun Gold, however, exhibited a significant decrease (14%) in total lycopene content compared with normal fruit (Table 4). Seedless fruit of Giza 1, Sugar Baby, Korgan and Jingxin had a significant increase in β-carotene content (44%, 27%, 33% and 33%, respectively) compared with normal fruit, while for Giza 21, Sun Gold and Sharmen no difference was observed (Table 4). In Crimson Sweet, however, the tendency was reversed, as the β-carotene content decreased in seedless fruit by 30% as compared with the normal fruit (Table 4).

Table 3

Growth characteristics of watermelon fruits developed using Giza 1 pollen irradiated with gamma rays (600 Gy) to pollinate each watermelon plant

Cultivars	Fruit type	Fruit weight (kg)	Flesh colour [a]	Thickness of rind (mm)	Days to maturity [b]	No. of seeds		
						Normal	Empty	Total
Giza 1	Normal	7.2	r 3.4	7.2	55.5	598	43	641±80 [c]
	Seedless	8.0**	r 3.5	7.1	55.2	0	10	10±1
Giza 21	Normal	5.0	r 4.0	8.3	57.2	491	90	581±46
	Seedless	5.8**	r 4.6**	9.6**	57.3	0	15	15±2
Crimson Sweet	Normal	3.3	r 3.4	10.9	55.0	353	122	475±43
	Seedless	3.7*	r 3.2	10.7	55.1	0	63	63±5
Sugar Baby	Normal	2.6	r 4.9	7.0	51.1	207	18	225±27
	Seedless	2.9*	r 4.7	7.2	51.0	0	25	25±3
Sun Gold	Normal	7.1	r 3.6	11.2	52.1	525	183	708±43
	Seedless	8.0**	r 3.5	13.3**	52.1	0	221	221±33
Korgan	Normal	6.8	r 3.0	16.9	56.0	381	90	471±61
	Seedless	6.9	r 3.5*	13.7**	56.1	0	104	104±13
Sharmen	Normal	5.9	r 3.1	10.9	60.3	499	114	613±86
	Seedless	5.0*	r 3.0	8.6**	60.0	0	76	76±10
Jingxin	Normal	4.8	r 3.2	11.0	53.5	430	88	518±57
	Seedless	5.1*	r 3.2	10.8	53.0	0	61	61±8

a: Red colour on a scale of 1 (light) to 5 (deep); b: From pollination to harvest.; c: Mean ± SE; *, ** Significant difference between normal and seedless fruit at $P<0.05$ or 0.01, respectively.

Table 4

Growth constituents of watermelon fruits developed using Giza 1 pollen irradiated with gamma rays (600 Gy) to pollinate each watermelon plant

Cultivars	Fruit type	Total sugar (%)	Lycopene content (μg)	β-carotene content (μg)
Giza 1	Normal	10.5	83	9
	Seedless	12.6**	102**	13**
Giza 21	Normal	10.3	102	6
	Seedless	11.4*	114*	5
Crimson Sweet	Normal	9.4	83	10
	Seedless	9.3	84	7**
Sugar Baby	Normal	10.0	110	8
	Seedless	11.1**	126*	11**
Sun Gold	Normal	10.6	79	5
	Seedless	11.3**	68**	5
Korgan	Normal	9.3	98	6
	Seedless	10.1*	100	8*
Sharmen	Normal	10.5	67	8
	Seedless	12.5**	81**	7
Jingxin	Normal	9.6	90	9
	Seedless	10.5*	108*	12**

*, ** Significant difference between normal and seedless fruit at $P<0.05$ or 0.01, respectively.

Experiment IV

In the case of both unirradiated and irradiated pollen, the pollen tube penetrated into one of the two synergids one day after pollination and sperm nuclei were normally discharged. At 2 to 5 days after pollination, a sperm nucleus and egg nucleus were attached to each other in the egg cell (Figs. 2a, 3a). The other sperm nucleus in the same embryo sac was adjacent to the 2 polar nuclei. Proembryo and globular-shaped embryo were formed 7 to 10 days after pollination with unirradiated pollen (Fig. 2b) and a heart-shaped embryo was observed at about 10 days (Fig. 2c). The embryo differentiated into cotyledons, radicle, epicotyl and hypocotyl by days 12 to 16, and subsequently developed into a mature embryo. In contrast, there was a slight delay in the development of the proembryo and globular-shaped embryo following pollination with irradiated pollen compared with unirradiated pollen (Fig. 3b). The shape of the globular embryo was deformed and there were fewer cells than in a normal embryo. The embryo failed to differentiate organ tissues and degenerated 10 to 22 days after pollination (Fig. 3c).

Discussion

Numerous studies have demonstrated that irradiated pollen can germinate on the stigma, grow within the style and reach the embryo sac, but cannot fertilize the egg-cell and the polar nuclei. However, irradiated pollen can stimulate the division of the egg cell, and thus induce parthenogenesis or the development of parthenocarpic fruit (Musial and Przywara, 1998). The response of pollen to irradiation varies greatly and is plant genotype- and dose-dependent (Brewbaker and Emery, 1962). Higher doses increase the frequency of parthenogenetic embryos, which can be explained by the 'Hertwig' effect, the production of maternal individuals with the use of male gametes treated with high doses of ionizing radiation (Pandey and Phung, 1982). In kiwifruit, haploidization was successfully obtained by inducing parthenogenesis through pollen irradiation (Chalak and Legave, 1997; Musial and Przywara, 1998). Pollen irradiated with gamma rays did not affect the fruit set. It was observed that the germination rate of pollen treated with 600 Gy was almost the same as that of the control (Sugiyama and Morishita, 2000). These results indicate that irradiated pollen can be used for producing seedless watermelon.

Triploids tend to show a longer growing period and later maturity than diploid ones (Kihara, 1958), and they sometimes display developmental defects such as hollow hearts and thick rinds. In contrast, such defects were not observed in the seedless watermelon produced using pollen irradiated with gamma rays for pollination, because diploid seedless watermelon can be produced using common diploid cultivars. The occurrence of empty seeds was recognized long ago in triploid watermelon. In diploid watermelons, when seedless watermelons are produced from cultivars with few normal seeds, the

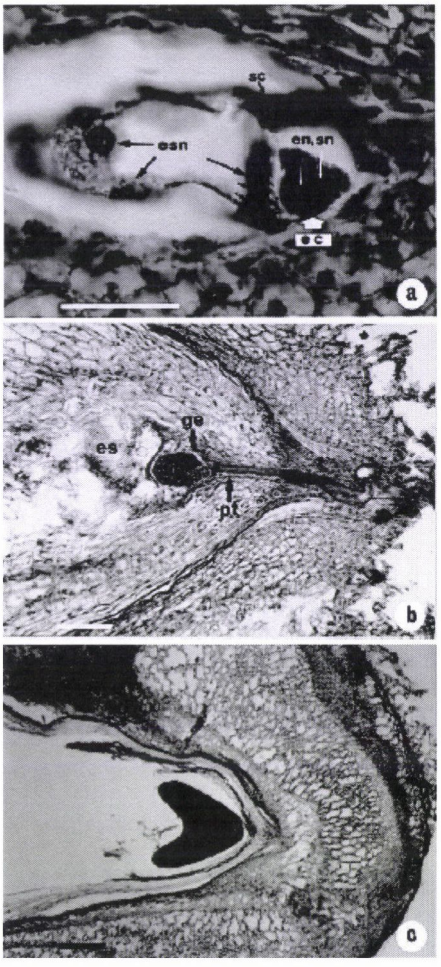

Fig. 2. Process of fertilization and embryo formation with unirradiated pollen

(a): Section of embryo sac on day 4 (96 h) after pollination with unirradiated pollen, showing an egg nucleus and sperm nucleus attached to each other in the egg cell and endosperm nuclei. The endosperm nuclei have expanded in the embryo sac. (b): Section of embryo sac on day 8, showing globular embryo. (c): Section of embryo sac on day 10, showing heart-shaped embryo. ec, egg cell; en, egg nucleus; es, endosperm; esn, endosperm nuclei; ge, globular embryo; pt, pollen tube; sc, synergid cell; sn, sperm nucleus. Horizontal bar: (a), 25 μm; (b), 50 μm; (c), 100 μm

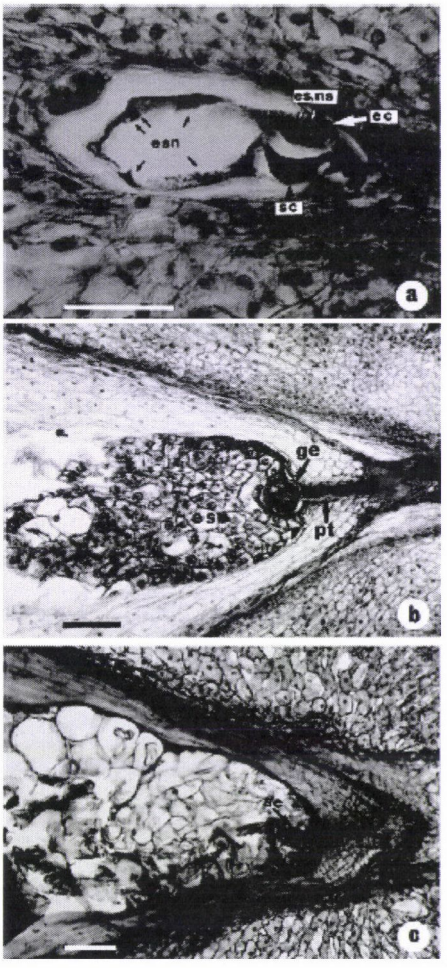

Fig. 3. Process of fertilization and embryo formation with irradiated pollen

(a): Section of embryo sac on day 4 (96 h) after pollination with irradiated pollen, showing an egg nucleus and sperm nucleus attached to each other in the egg cell. The endosperm nuclei have spread in the embryo sac. (b): Section of embryo sac on day 8, showing globular embryo. (c): Section of embryo sac on day 20, showing an aborted embryo. ae, aborted embryo; ec, egg cell; en, egg nucleus; es, endosperm; esn, endosperm nuclei; ge, globular embryo; pt, pollen tube; sc, synergid cell; sn, sperm nucleus. Horizontal bar: (a), 25 μm; (b), 50 μm; (c), 100 μm

fruits contain few empty seeds. However, when seedless watermelons are produced from cultivars with very few seeds, the number of empty seeds may not necessarily decrease. Also, there was a high correlation between the size of normal seeds and empty seeds (Sugiyama and Morishita, 2000). Coloured empty seeds were sometimes observed in seedless fruits. A coloured empty seed is produced when the seed coat is black or brown. Therefore, it is preferable to use cultivars with a white seed coat to produce seedless watermelon. The number of empty seeds is influenced by the temperature, and the colour of the empty seeds also tends to become deeper when the temperature is high (Watanabe et al., 2002). It is necessary to analyse the effect of environmental factors on seed formation. Also, the results indicate that seedless watermelon cultivars have a significant increase in total sugar and carotenoid (lycopene and β-carotene) content, providing an important source of phytonutrients in the diet (Perkins Veazie et al., 2004). In a previous study on watermelon, the discharge of sperm nuclei occurred 2 days after pollination with normal pollen (Buttrose and Sedgley, 1979). In the present study, a similar finding was observed after pollination with irradiated pollen. Although embryonic development was reported to occur in watermelon in pollinated, but not in unpollinated (Buttrose and Sedgley, 1979) or auxin-induced parthenocarpic ovules (Sedgley et al., 1977), it was assumed in the present work that after sperm cell discharge fertilization occurred with pollen irradiated with gamma rays as well as with unirradiated pollen. It is generally recognized that the irradiation of pollen with γ-rays may induce genetic abnormalities. The abortion of embryos after pollination with pollen irradiated with gamma rays may be due to chromosomal abnormalities induced by gamma rays in the generative nucleus (Vaijapurkar et al., 2001). It is thus necessary to determine whether all or part of the chromosomes in the sperm nuclei are involved in nuclear fusion.

In Japan, the main reason triploid seedless watermelons are not popular is that triploids have a longer growing period and later maturation date than diploid ones. Occasionally, defects such as hollow hearts, short shelf-life or low sugar contents have also been observed in triploid watermelon fruits. These defects were not present in the seedless watermelon produced by pollination with gamma-irradiated pollen. Therefore, the high quality of diploid fruit is retained in seedless fruit. This method can also be applied to other fruits (lemon, orange, persimmon, etc.). In Japan, irradiated pollen is now available on the market. If irradiated pollen can be mass-produced, it will become cheap.

It is suggested that the seedless fruit induced by gamma rays had a beneficial effect in increasing the quantity and quality of watermelon yield via increases in the carotenoid, total sugar content and fruit weight. Also, in some cultivars there is a tendency for the thickness of the rind to decrease.

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INVESTIGATION OF FACTORS INFLUENCING THE REGENERATION EFFICIENCY OF *Rubus* SPECIES

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An efficient regeneration system is described based on the use of several genotypes and combining different cytokinins in the regeneration process. Optimal regeneration efficiency can be obtained if the factors affecting regeneration are examined with special attention to the maintenance of the stock plants, the composition of the medium, and the pre-treatment. The maintenance of stock plants proved to be optimal if the plants were kept on modified LS medium supplemented with 0.125 mg L^{-1} 6-benzylaminopurine (BAP) and 0.01 mg L^{-1} indole-3-butyric acid (IBA) in large vessels. Pre-treatment was found to increase the regeneration efficiency. Placing the leaves on to medium containing 1.5 mg L^{-1} BAP-riboside and 0.1 mg L^{-1} thidiazuron (TDZ) without wounding, and keeping them in the dark for 6 days gave the best results. The highest regeneration rate was observed on medium containing MS salts with B5 vitamins complemented with 20 g L^{-1} glucose, 3 mg L^{-1} BAP-riboside, 0.2 mg L^{-1} TDZ and 0.2 mg L^{-1} IBA. This system made it possible to achieve regeneration in each of the varieties examined, though to different extents.

Abbreviations: BAP: 6-benzylaminopurine, FeEDDHA: ethylenediamine di-2-hydroxy-phenyl acetate ferric salt, FeNaEDTA: ethylenediaminetetraacetic acid ferric-sodium salt, IBA: indole-3-butyric acid, TDZ: thidiazuron

Key words: *Rubus*, *in vitro* regeneration, pre-treatment, cytokinins, stock plant maintenance

Introduction

It is a prerequisite for genetic engineering to have a reliable *in vitro* regeneration system. *Rubus* species (raspberry, blackberry and their hybrids) were classified as recalcitrant for a long time, but several papers have now reported successful regeneration (McNicol and Graham, 1990; Fiola et al., 1990; Cousineau and Donnelly, 1991; Turk et al., 1994; Graham et al., 1997). However, the number of teams that have been able to genetically modify this species is still limited (McNicol and Graham, 1989; Hassan et al., 1993; Mathews et al., 1995; Mezzetti et al., 2004).

In previous experiments a regeneration system for four different *Rubus* cultivars (Kálai et al., 2005) was found to be inadequate for transformation purposes. During genetic modification only a small percentage of the regenerated cells proved to be transgenic, so an efficient regeneration system resulting in a large number of plantlets needs to be elaborated.

The aim of the present work was to examine the factors necessary to develop protocols with high regeneration frequencies from leaf and petiole explants of different *Rubus* genotypes. According to previous observations (Reed, 1999; Mezzetti et al., 1997) great differences can be found among the genotypes and it is almost impossible to develop a repeatable protocol suitable for all of them. For this reason the number of genotypes was extended to eight. A number of experiments were performed to improve the regeneration method. Factors affecting regeneration were examined with special attention to the maintenance of the stock plants, the composition of the media, and leaf pre-treatment. The effect of wounding and the type of explants was also investigated.

Materials and methods

Plant material

Different *Rubus* varieties were used in the experiments, two of which, 'Hull Thornless' blackberry (Maryland, USA) and 'Malling Exploit' raspberry (East Malling, UK) were approved and registered in Hungary in the early eighties. The hybridberry (blackberry × raspberry) 'Fertődi Bőtermő', the raspberry varieties 'Fertődi Kármin', 'Fertődi Vénusz', 'Fertődi Zamatos' and 'Fertődi Zenit' and the prospective variety 'Fertődi 6585' were selected at the Fertőd Research Institute For Fruit Growing. The basic, virus-free shoot culture material is maintained in the gene bank of this institute.

Micropropagation

Stock plants were selected from a virus-free plantation and sterile cultures were initiated from meristems. Shoot cultures were maintained on three different basic media, namely MS salts and vitamins (Murashige and Skoog, 1962), MS salts and B5 vitamins (Gamborg et al., 1968) or modified LS medium (James et al., 1980). The LS medium was modified as regards the ferric content, since 50 mg L^{-1} FeNaEDTA was used in the present experiments. All the media were supplemented with 20 g L^{-1} sucrose and 0.01 mgL^{-1} IBA. To obtain the best rate of proliferation and the best quality plants, different concentrations of BAP were tested. BAP was chosen because most authors recommend this cytokinin for maintenance and proliferation. In order to find the most appropriate vessel and mode of aeration the stock plants were maintained in different containers: plastic boxes or round plastic vessels with a breathing strip, designed for tissue cultures (products of Duchefa), in 100 mL or 500 mL Erlenmeyer flasks with cotton plugs.

Regeneration experiments

The three apical leaves were excised from 4-week-old shoots grown on proliferation medium. Leaves with a small petiole segment were detached and two or three transversal cuts were made across the leaf midrib before the leaves were placed adaxial side down on the medium. Petiole explants were also tested: 0.5–1 cm long petiole segments were placed on the surface of the medium.

The effect of leaf pre-treatment on shoot induction was also investigated. Leaves from 5-week-old proliferating shoots were placed on medium containing MS salts and B5 vitamins supplemented with 20 g L^{-1} glucose (MSB5gl medium) and various hormone combinations. The cultures were incubated in the dark for 5, 6 or 7 days.

Various media were used in the regeneration experiments, as summarized in Table 1.

In most cases 80 explants/variety/hormone combination were used in three replications. Only rooted shoots were counted. Several shoots are usually obtained from each explant, but this effect was not considered in the statistics.

Table 1
Hormone combinations used in the regeneration experiments

Code of medium	Cytokinins (mg L^{-1})						Auxins (mg L^{-1})	
	BAP	BAP-rib	TDZ	Kin	2-ip	Zea	IBA	IAA
1	2						0.1	
2			2.2				0.1	
3	1	1	1				0.1	
4				1			0.1	
5					1		0.1	
6						1	0.1	
7	1	1					0.1	
8			1				0.1	
9	1		1				0.1	
10	3						0.2	
11		2					0.1	
12		1	1				0.1	
13		3					0.2	
14		3	0.2				0.2	
17		1.5	0.2				0.2	
19		1	0.5					0.2
20		2	0.1					0.2
21		3	0.2				0.2	
22		3	0.2					0.2
24		2	0.2				0.2	
26		2	0.1					0.2
27		3	0.1					0.2
28		1.5	0.1				0.2	
29		2	0.1				0.2	

The basic medium in all cases consisted of MS macro- and microelements + B5 vitamins + 20 g L^{-1} glucose. Abbreviations: BAP: benzylaminopurine; BAP-rib: benzyilaminopurine-riboside; TDZ: thidiazuron; Kin: kinetin; 2-ip: isopentenyl-adenine; Zea: zeatin; IBA: indolyl-butyric acid; IAA: indolyl-acetic acid

Results and discussion

It was found that the best quality stock plants could be maintained on modified LS medium (James et al., 1980) and that higher ferric content (50 mg L^{-1} FeNaEDTA) and lower sucrose (20 g L^{-1}) content improved the quality of the stock plants. Another ferric source was also examined (FeEDDHA), which

had a slightly positive effect on stock plant quality, but had no effect on regeneration, as also reported by Zawadzka and Orlikowska (2006). The ideal hormone concentration was 0.125 mg L^{-1} BAP and 0.01 mg L^{-1} IBA. Lower hormone concentrations resulted in a low proliferation rate, while at higher concentrations the leaves developed were too small.

It became clear that stock plants require vessels with a large capacity. In small vessels the leaves of the plants became soft, making them unsuitable for *Agrobacterium*-mediated transformation. For this reason 500 mL Erlenmeyer flasks and round vessels from Duchefa were used for maintaining stock plants.

The effect of pre-treatment has rarely been reported. Swartz et al. (1990) found that pre-treating proliferating shoots with colchicine and TDZ or various cytokinins also improved the percentage of organogenesis, although the best response was observed for colchicine. Meng et al. (2004) also found that a pre-treatment medium containing TDZ optimized the shoot formation of blackberry. In both of these experiments the shoot cultures were pre-treated in the last subculture prior to excising the leaves for shoot induction

The positive effect of leaf pre-treatment was also observed in the present investigations. The combination of two cytokinins: 1.5 mg L^{-1} BAP-riboside and 0.1 mg L^{-1} thidiazuron (TDZ), proved to be the most efficient for shoot induction. When the effect of dark treatment was investigated, 6 days of incubation in the dark gave the best results. During this period the veins and petioles of the leaves became swollen (Fig. 1A).

The effect of pre-treatment depended on the variety, but, as shown in Table 2, it was positive for all the varieties examined: the regeneration rate increased 4–23 times when pre-treatment was applied.

In earlier works it was suggested that halved leaves or leaves with 2–3 transversal cuts on the midrib should be used as explants. This was confirmed by the observations of Fiola et al. (1990), Mezzetti et al. (1997) and Turk et al. (1994). Although both types of explants are suitable for regeneration, the extent of wounding caused the explants to die too quickly for second or third regenerants to be collected. It was also observed that in many cases regeneration occurred on the petioles (Fig. 1B), but since long petioles do not come into contact with the bacteria during cocultivation with an *Agrobacterium* suspension, the plantlets developing on the petioles may remain untransformed. As it was also found that detached petiole segments were not suitable for regeneration, another source of explants was sought. Leaves on which the veins and petioles became swollen during pre-treatment were used as explants. The petioles were cut from the leaf leaving a very short stump and one small transversal cut was made on the swollen midrib. These explants were placed adaxial side down on the medium.

The best medium for regeneration was MSB5gl supplemented with 3 mg L^{-1} BAP-riboside, 0.2 mg L^{-1} TDZ and 0.2 mg L^{-1} IBA. Regeneration could be achieved for all the varieties using this method (Fig. 1C and D).

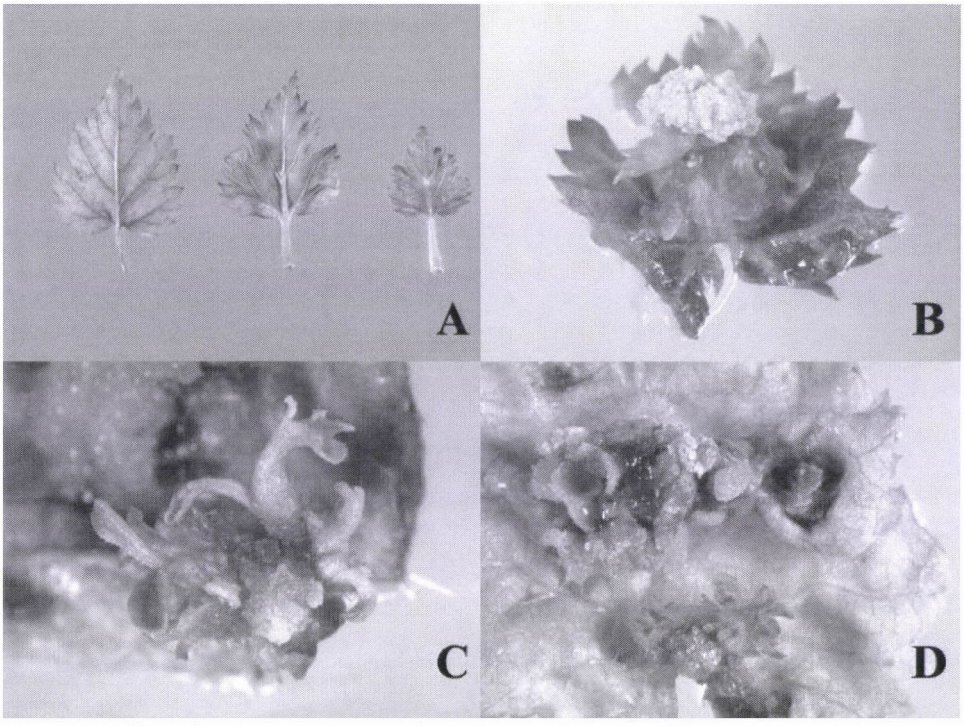

Fig. 1. A. Effect of pre-treatment on raspberry leaves: the veins of the leaf on the left have not become swollen, unlike those of the leaves in the middle and on the right, which are more suitable for regeneration experiments. B: Regeneration on the petiole. C: Group of regenerants on cut petioles of the cultivar Fertődi Vénusz. D: Regenerants on Zamatos explants

Table 2
Regeneration rate (number of new shoots/explant) in the experiment designed to examine the effect of pre-treatment

Varieties	Fertődi Kármin	Fertődi Vénusz	Fertődi Bőtermő
Without pre-treatment	0.16	0.43	0.15
With pre-treatment	1.69	1.02	3.45

To the best of our knowledge, this is the first time that a combination of cytokinins has been applied. The regeneration efficiency was improved by applying pre-treatment. This reliable regeneration method could be a good basis for developing a transformation system for various *Rubus* species.

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STUDY OF ANDROGENESIS AND SPONTANEOUS CHROMOSOME DOUBLING IN BARLEY (*Hordeum vulgare* L.) GENOTYPES USING ISOLATED MICROSPORE CULTURE

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This research aimed to study the androgenesis and spontaneous chromosome doubling of five barley genotypes using an isolated *in vitro* microspore culture technique, involving a completely randomized design (CRD) with three replications. Statistical analysis of embryogenesis and cytogenetic results showed that genotype had a significant effect on haploid embryogenesis ($P<0.01$) and on spontaneous chromosome doubling ($P<0.05$). The genotype Igri was found to have the highest potential to produce haploid embryos (1577 embryos from 100 anthers), followed by the genotypes Boyer/Rojo, Afzal/Turkman/Kavir, Ashar/Hebo and Agrigashar/Matico with 369, 304, 278 and 150 embryos from 100 anthers, respectively. The highest percentage of spontaneous chromosome doubling (76%) was observed for the genotype which had the lowest embryogenesis (Agrigashar/Matico) and the lowest (65%) for the genotype with the highest androgenic capacity (Igri). Microspore embryogenesis also showed comparatively higher genotypic (109.2) and phenotypic (109.5) coefficients of variation, heritability (99.62) and genetic advance (1206.77), indicating the pre-dominance of additive gene action in the control of this character in the material studied. Estimates of genetic parameters (PCV, GCV and heritability) for microspore embryogenesis were higher than for spontaneous doubled haploids. These results indicated that selection for androgenic capacity would be more effective than for spontaneous doubled haploids. The findings showed a negative relationship ($r= -0.68$) between embryogenesis and spontaneous chromosome doubling in the barley genotypes studied. All the large embryos used had high regenerability and good plantlet formation.

Key words: haploid embryogenesis, spontaneous chromosome doubling, microspore culture, barley (*Hordeum vulgare* L.), heritability, genetic advance

Introduction

The method of *in vitro* plant breeding has been used for improving various traits in many crops (Taji et al., 2002; Hagio et al., 1995; Kahrizi et al., 2007a, b). The production of doubled haploid (DH) plants through the induction of

androgenesis is a promising and convenient alternative to conventional selfing techniques for the generation of pure lines for breeding programmes (Seguí-Simarro and Nuez, 2008). Isolated microspore culture is a powerful tool for producing haploid and doubled haploid plants for *in vitro* plant breeding. This technique may allow the faster production of new varieties than using conventional breeding methods and has been successfully employed in many crop plants (Kasha et al., 1997; Kahrizi, 1998; Nägeli et al., 1999; Kahrizi, 2001).

The regeneration potential of the culture is dependent upon the donor plants, staging, pre-treatment, isolation and culture medium (Kasha et al., 1997; Kahrizi et al., 2000). Barley is important as a global crop and as a leading modell plant for isolated microspore culture and cereal transformation studies (Jähne and Lörz, 1995). Haploid plants must be chromosome doubled to restore fertility for use in plant breeding. The chromosome doubling of microspore-derived plantlets and calli is a critical step in haploid breeding programmes (Mozafari et al., 1997).

Colchicine is the compound most commonly used to double the chromosomes in androgenesis experiments. It has been applied to regenerated plants after transfer to soil (Inagaki, 1985), or initially in the *in vitro* microspore culture substrate (Barnabás et al., 1991; Hansen and Anderson, 1998a). Colchicine, however, is toxic, carcinogenic and expensive (Hansen and Anderson, 1998b).

The spontaneous chromosome doubling rate in microspore-derived wheat plants is only 15–25% (Navarro-Alvarez et al., 1994), which necessitates artificial chromosome doubling. It has been reported that in barley spontaneous chromosome doubling constituted 70–80% of the regenerated population and only 15–20% plantlets were haploids (Tiwari and Rahimbaves, 1992). The duplication of the haploid genome of androgenic plants has been thought to occur through three mechanisms: endoreduplication, nuclear fusion and c-mitosis (Seguí-Simarro and Nuez, 2008).

Plant breeding programmes depend on the knowledge of key traits, the genetic systems controlling their inheritance, and the genetic and environmental factors that influence their expression (Kester et al., 1977). In the present study the inheritance, genotypic coefficient of variation (GCV) and phenotypic coefficient of variation (PCV) of the characters microspore embryogenesis and spontaneous doubled haploid production were considered.

The present paper deals with the effect of genotype on isolated microspore culture and spontaneous chromosome doubling in barley (*Hordeum vulgare* L.) and describes the successful production of barley doubled haploids without applying colchicine or other chemical agents.

Materials and methods

Microspore donor plants

Five barley genotypes, consisting of a cultivar Igri and four F_3 populations of Afzal/Turkman//Kavir, Ashar/Hebo, Boyer/Rojo and Agrigashar/Matico, were used to investigate isolated microspore culture and spontaneous chromosome doubling.

The growth conditions of donor plants are critical to the success of microspore culture and were the same as described previously (Kahrizi, 2001). Donor plants were grown in a greenhouse with a photoperiod of 16/8 hours (light/dark), a temperature of 15°C/12°C (day/night), 80% relative humidity and a light intensity of 60–80 μmol $m^{-2}s^{-1}$ for 6–8 weeks.

Isolated microspore culture and embryo subculture

Immature spikes were harvested when the majority of the microspores were at the mid-to late uninucleate stage and the leaf sheath was then surface-sterilized with 70% (700 ml l^{-1}) ethanol for 1 min before pre-treatment. The system of staging microspores described previously was used (Kahrizi, 2001). The microspores were isolated from the anthers using the maceration method described previously by Kasha et al. (1991). The anthers were removed and placed in 0.3 M mannitol solution for 4 days at 25°C. The liquid collected from filtration (through two layers of Whatman No. 2 filter paper, 42.5 mm, under a vacuum) was centrifuged for 10 min at 700 rpm in 40 ml glass graduated centrifuge tubes (Kimax, San Fransico) to gently pellet the microspores.

The collected microspores were resuspended in liquid FHG medium containing 62 g l^{-1} maltose monohydrate, 730 mg l^{-1} glutamine, 1 mg l^{-1} BAP and 10 mg l^{-1} PAA. The microspores were plated at a density of 4×10^4 ml^{-1}. The cultures were incubated at 25°C in the dark for 3 weeks. At this stage, embryo-like structures (ELS) emerged. Visible embryos were counted, and large ones (≥1.5 mm in diameter) were subcultured to modified MS solid medium (Kasha et al., 1997) and incubated at 25°C under a 16/8 hour low light/dark cycle, where they were kept until they had developed green leaves. At the 3–5-leaf stage, the plantlets were transferred to MS regeneration medium (hormone-free) in magenta boxes.

After root formation on the above medium the plantlets were transferred to a potting mix supplemented with liquid fertilizer. The plants were grown in a misting chamber under the growth conditions used for donor plants, as described above. Under these conditions the plants were established rapidly. They were then transferred to the greenhouse and allowed to flower and set seed. Cytogenetic tests were carried out using the method of Mujeeb-Kazi and Miranda (1985) and the ploidy level of the plants was determined. The coefficient of variability was calculated using the methodology suggested by Burton and de Vane (1953). Heritability and genetic advances were calculated with the formula suggested by Johnson et al. (1955).

Statistical analysis

Analysis of variance was carried out in order to determine the significance of differences between the genotypes. Duncan's multiple range test was used to compare the mean performance of the genotypes for androgenetic embryogenesis and spontaneous chromosome doubling. All data were normalized by transformation using the Arcsin $\sqrt{x}$ function.

Results

The results of ANOVA for the studied traits showed that the effect of genotype was significant for *in vitro* microspore embryogenesis ($P < 0.01$) and spontaneous chromosome doubling ($P < 0.05$) (Table 1). The genotype Igri had the highest frequency of embryoid formation (1577 embryoids from 100 anthers) and Agrigashar/Matico the lowest (150.3 embryoids from 100 anthers), while

the other genotypes, Afzal/Turkman/Kavir, Ashar/Hebo and Boyer/Rojo, had similar levels of embryoid formation (Table 2). Anthers containing microspores at the mid- to late uninucleate stages of development were cultured in mannitol liquid medium. The cultured microspores formed multicellular structures which later developed into embryo-like structures (ELS) (Fig. 1A,B) and all the large embryos were regenerated (Fig. 1C).

The lowest percentage of spontaneous chromosome doubling (SCD) was observed for the genotype Igri (65%), which had the highest microspore embryogenesis, and the highest for the genotype Afzal/Turkman/Kavir (76%), which had the lowest microspore embryogenesis.

The present study further revealed that spontaneous doubled haploids (2n=2x=14) comprised 65 –76% of the regenerated population and only 20–30% plantlets were haploid (n=x=7) (Fig. 2). The results showed a negative correlation between microspore embryogenesis and spontaneous chromosome doubling (r= –0.68). The doubled haploid plants were completely fertile and were able to flower and set seed.

Table 1

Analysis of variance for androgenetic embryoid formation (Number of haploid embryos produced from 100 anthers, E/100A) and spontaneous doubled haploid formation (SDH) in five barley genotypes

Source of variation	Df	E/100A	SDH
Genotype	4	1034789**	65.1*
Error	10	1301.87	15.4
CV		6.73%	5.51%

*, ** Significant at the 5% and 1% level of probability

Table 2

Mean performance of five barley genotypes for embryoid formation and spontaneous doubled haploid percentage

Genotypes	Embryogenesis means	SDH percentage
Afzal/Turkman/Kavir	304.0 b	72 a
Ashar/Hebo	287.3 b	75 ab
Boyer/Rojo	369.3 b	68 abc
Agrigashar/Matico	150.3 c	76 bc
Igri	1577.0 a	65 c

In each column, data followed by the same letter are not significantly different

Table 3

Variability parameters for microspore embryogenesis and spontaneous doubled haploid production in barley

Trait	GCV	PCV	ECV	h^2_b	GA	GG
Microspore embryogenesis	109.2	109.5	6.7	99.62	1206.77	224.58
Spontaneous doubled haploid production	5.70	7.94	5.51	51.83	6.04	8.48

GCV: Genotypic coefficient of variation; PCV: Phenotypic coefficient of variation; ECV: Environment coefficient of variation; h^2_b : Heritability (%) (broad sense); GA: Genetic advance; GG: Genetic gain (%)

The estimates of genetic parameters (PCV, GCV and heritability) for microspore embryogenesis were higher than those for spontaneous doubled haploid (Table 3). The increased genetic variability of microspore embryogenesis provides great scope for further selection.

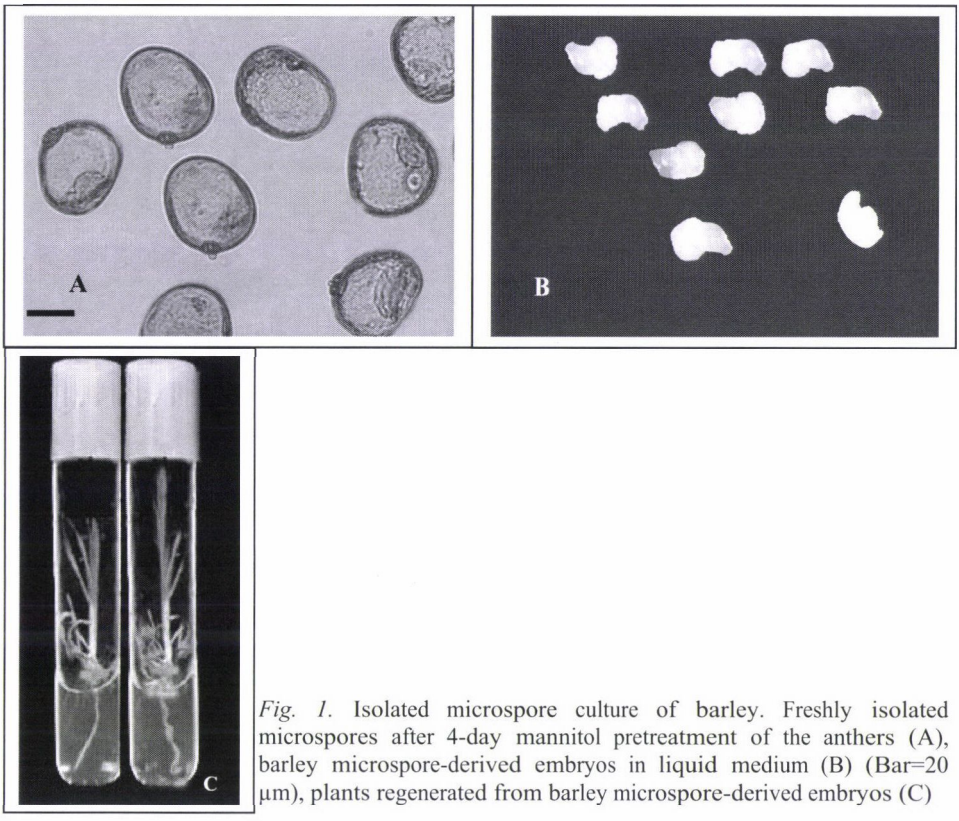

Fig. 1. Isolated microspore culture of barley. Freshly isolated microspores after 4-day mannitol pretreatment of the anthers (A), barley microspore-derived embryos in liquid medium (B) (Bar=20 μm), plants regenerated from barley microspore-derived embryos (C)

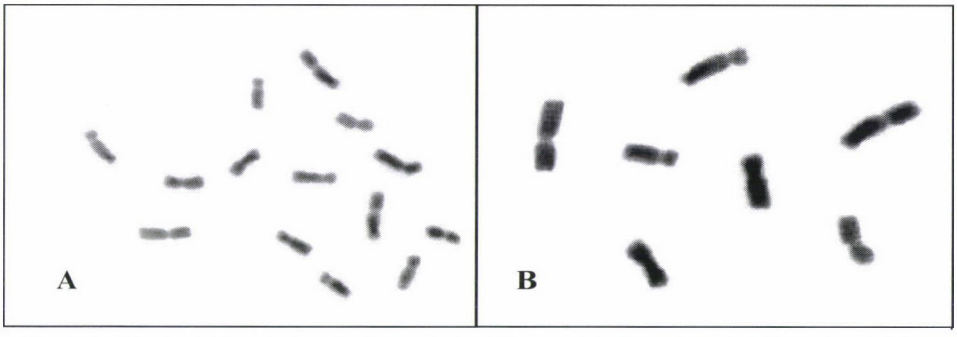

Fig. 2. Cytogenetic test for androgenetic plantlets. The majority of plantlets were spontaneous doubled haploids (A) with a low percentage of haploids (B)

The heritability estimates were high (>99%) for the character microspore embryogenesis, while a comparatively low value of heritability was observed for the character spontaneous doubled haploid production (51.83%). The high value of broad sense heritability for microspore embryogenesis revealed that this character is less influenced by the environment and there could be greater correspondence between phenotypic and breeding values.

The expected genetic advance (GA), expressed as a percentage of the mean genetic gain, significantly varied from 1206.77 for microspore embryogenesis to 6.04 for spontaneous doubled haploids.

Discussion

The genotype of the donor material may affect androgenic embryogenesis, plant regeneration and albinism in barley (Carlson, 1998). The results of the present work showed that the genotype significantly affected embryoid formation (Table 1). This is in agreement with the results of Castillo et al. (2000), while Li and Devaux (2003) and Kasha et al. (2004) reported that there was no significant difference in embryo induction between genotypes.

Doubled haploids are excellent materials for plant genetic research, plant breeding and transformation (Kahrizi, 2001). Colchicine is the antimitotic agent most commonly used for doubling the chromosomes (Inagaki, 1985), but it has relatively low efficiency for plant microtubules, has carcinogenic effects in humans and is expensive (Hansen and Anderson, 1998b). In the cytogenetic section of this research the focus was on the chromosome doubling of haploids without applying antimitotic agents and on the effect of genotype on chromosome doubling.

The results showed that there was no significant difference between the genotypes for spontaneous chromosome doubling, with a mean doubling frequencies 65–76% (Table 2). The regenerated plants were completely fertile doubled haploids (Hoekstra et al., 1992; 1993; Hu and Kasha, 1999). The high frequency of completely fertile plants indicates that chromosome doubling must occur very early, most likely at the time of induction (Kasha et al., 2001; Seguí-Simarro and Nuez, 2008).

The mechanism of chromosome doubling is not thoroughly clarified and the relationship to the influence of pre-treatments is obscure, with endoreduplication and nuclear fusion as the most likely methods. C-mitosis, such as occurs during colchicine treatment, may result in a simple restitution nucleus with a doubled chromosome number. In *Datura*, it was proposed that both endoreduplication and nuclear fusion were involved in chromosome doubling and that the combination of both methods could explain the ploidy levels obtained, which were higher than diploid (Sunderland, 1974; Sunderland et al., 1974; Seguí-Simarro and Nuez, 2008). Nuclear fusion was described as occurring when two nuclei synchronously entered into division, formed a

common metaphase plate and spindle and resulted in two nuclei, each with more than one set of chromosomes (Sunderland, 1974). If one or both of the nuclei had undergone endoreduplication prior to nuclear fusion, triploid or higher ploidy level plants could be formed. Both the stage of the microspore when collected for pre-treatment and the pathway of nuclear development have also been considered to influence the frequency of doubling (Sunderland, 1974). It was concluded that microspores collected at uninucleate stages 1–3 (early, mid and late, respectively) resulted in mostly haploid and doubled haploid plants, while those collected at later stages (4–6, mitosis and binucleate) resulted in mostly doubled haploids as well as some triploid and tetraploid plants. It has also been demonstrated in wheat that the pre-treatment method influences the pathway along which the nuclei develop (Hu and Kasha, 1999).

A switch from normal gametophytic to embryogenic (sporophytic) development can be induced by the pre-treatment of anthers or spikes. Pre-treatment also influences the stage of the microspores. Hu and Kasha (1999) found that uninucleate microspores of wheat completed the first mitotic division during both 28-day cold pre-treatment and 6–7 days of 0.4 M mannitol pre-treatment at 28°C (Hu and Kasha, 1999). It was also reported that spike pre-treatment combining 0.4 M mannitol solution and cold pre-treatment for 4 d in wheat essentially blocked the mitotic division of the nucleus, holding all microspores at the same stage during pre-treatment, and also resulted in the formation of large numbers of true embryo-like structures (ELS) (Hu and Kasha, 1999).

The progress of a breeding programme is conditioned by the magnitude and nature of the genotypic and non-genotypic variation in the various characters. The magnitude of heritability indicates the reliability with which a genotype can be recognized by its phenotype expression and the effect that environment has on its expression. The heritability estimates for different characters depend upon the genetic makeup of the breeding materials studied. Therefore, knowledge about these values in the materials in which breeders are interested is of great significance. High heritability estimates indicate that selection for these characters will be effective, as they are less influenced by environmental effects.

Heritability estimates have been found to be useful in indicating the relative value of selection based on the phenotypic expression of different characters. Johnson et al. (1955) reported that heritability values along with estimates of genetic gain (GG) were more useful than heritability alone in predicting the effect of selection. High heritability estimates associated with high genetic gain were obtained for the character microspore embryogenesis, which indicated that selection for this character would be effective. High heritability values followed by high genetic advance indicate the presence of additive gene action (Johnson et al., 1955).

The difference between GCV and PCV for spontaneous doubled haploid production was moderate, indicating the moderate influence of the environment on the expression of this character, which can be induced by pre-treatment of the anthers with mannitol (Hu and Kasha, 1999).

Conclusions

Studies in barley microspore culture showed overall a significantly different response between genotypes for the formation of embryo-like structures (ELS). All the large embryos obtained were regenerated to plantlets. This research also demonstrated that the ploidy level of barley can frequently be doubled without using colchicine or any other antimitotic agent, independently of the genotype.

This study suggests that genotypic variation could be used to make a better estimate of the effect of genotype on androgenesis and spontaneous chromosome doubling. The utilization of other medium ingredients and plant growth regulators may be effective in isolated microspore culture to improve the spontaneous chromosome doubling frequency in barley.

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INFLUENCE OF 8-HYDROXYQUINOLINE SULPHATE AND SUCROSE TREATMENTS ON THE POST-HARVEST QUALITY OF CUT FLOWERS OF *Strelitzia reginae* AND *Hippeastrum vittatum*

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This investigation was carried out to study the effect of 100, 200 and 300 ppm 8-hydroxyquinoline sulphate (8-HQS) and 5 and 10% sucrose treatments on the vase life and post-harvest quality of cut flowers of *Strelitzia reginae* Ait. and *Hippeastrum vittatum* Herb. cv. Apple Blossome. All possible combinations of 8-HQS and sucrose were tested. The treatments were applied as holding solutions, and control flowers were held in distilled water till the end of the experiment. All the treatments significantly increased the vase life and number of open florets of *Strelitzia reginae* cut flowers compared to the control. Applying 8-HQS and sucrose treatments in both seasons improved the vase life and floret longevity of *Hippeastrum vittatum* cut flowers. In addition, the percentage of fresh weight gain from the initial weight and the carbohydrate content were also enhanced in both cut flower crops. In order to obtain the highest post-harvest quality of *Strelitzia reginae* Ait. and *Hippeastrum vittatum* Herb. cv. Apple Blossome cut flowers, treatment with 200 ppm 8-HQS + 10% sucrose was recommended.

Key words: 8-HQS, sucrose, vase life, carbohydrate, *Strelitzia reginae*, *Hippeastrum vittatum*

Introduction

The bird of paradise plant (*Strelitzia reginae* Ait. family *Strelitziaceae*) is one of the most important cut flower crops in Egypt. It is ranked high in the floral market and is also used in landscape design. The flowers exhibit irregular and incomplete floret opening. In addition, the florets wilt and brown within a few days of harvest. The challenges encountered by growers of tropical flowers like *Strelitzia reginae* mostly involve controlling flowering and ensuring adequate vase life (Pizano, 2005).

Amaryllis (*Hippeastrum vittatum* Herb., cv. Apple Blossome) is a well-known pot and garden plant and is also used as cut flowers. Native to South America and South Africa, amaryllis is a member of the family *Amaryllidaceae*,

with large bulbs producing spectacular trumpet-shaped blooms on hollow stalks (Schmidt, 2003). The flowering season of amaryllis in Egypt is short. The spikes appear from mid-April for 3 or 4 weeks and its vase life is about one week. A major cause of quality deterioration in these cut flowers is the blockage of xylem vessels by microorganisms that accumulate in the vase solution or in the vessels themselves. When the stem is blocked, continuing transpiration results in a net loss of water in flower and stem tissues. 8-hydroxyquinoline sulphate (8-HQS) is a very important germicide in the preservatives used in the floral industry (Nowak and Rudnicki, 1990). It acts as an anti-microbial agent (Ketsa et al., 1995) and also increases water uptake (Reddy et al., 1995).

Applying sucrose treatment significantly increased the number of open florets/inflorescence, floret vase life and inflorescence duration of *Strelitzia reginae* cut flowers (El-Mokadem et al., 1994; El-Saka et al., 1995). Pulsing *Strelitzia reginae* cut flowers with sucrose significantly increased the number of open florets as well as flower longevity (Finger et al., 2003). Reddy et al. (1995) reported that pulsing *Strelitzia reginae* cut flowers with 8-hydroxyquinoline citrate plus sucrose resulted in the maximum cumulative water uptake, maximum cumulative transpiration loss of water, lowest cumulative loss in weight, opening of maximum number of flowers and longest vase life. Pulsing treatment of *Strelitzia reginae* spikes with 8-HQS plus sucrose increased the number of open florets and prolonged the vase life (Hassan et al., 2008).

El-Saka et al. (2002) found that both the vase life and floret longevity of *Hippeastrum vittatum* flowers were significantly increased as a result of pulsing treatment with 8-HQS combined with sucrose. Tuberose flowers treated with 8-HQS + sucrose lasted 16 days compared to 8 days for the control. The water uptake was also improved by this treatment (Reddy et al., 1995). Holding solutions containing 8-HQS + sucrose improved water consumption, reduced the respiration rate and physiological loss in weight and extended the vase life of freesia flower stems (Kwon and Kim, 2000), gladiolus spikes (Beura and Singh, 2001), dendrobium flowers (Dineshbabu et al., 2002) and dahlia cut flowers (Abdel-Kader et al., 2004). Holding solutions containing 8-HQS and sucrose increased the water uptake, fresh weight and carbohydrate content and prolonged the vase life of various flower crops (Ichimura et al., 1999; Kim and Lee, 2002; Hassan et al., 2003; 2004).

Compared to most temperate flowers, there is a need for greater understanding of the morphological and physiological factors that limit the vase life of *Strelitzia reginae.* In addition, there is limited information in the literature concerning the comparative effectiveness of different chemicals on the post-harvest quality of *Hippeastrum vittatum* flowers. The aim of this study was to investigate the effect of 8-HQS and sucrose treatments on the post-harvest quality of *Strelitzia reginae* and *Hippeastrum vittatum* cut flowers.

Materials and methods

The flowers used in this experiment were obtained from the experimental farm of the Faculty of Agriculture, Tanta University, Tanta. Cut flowers were immediately brought to the laboratory of the Horticulture Department, Faculty of Agriculture, Tanta University. Bird of paradise flowers were harvested from the field during the November 2006 and 2007 seasons when one floret was fully opened and were trimmed to a length of 75 cm. The spikes of amaryllis were harvested during the April 2006 and 2007 seasons when the colour of the flower was shown but the spathe was still tight and the spikes were trimmed to 50 cm.

Each kind of flower was treated separately with 8-hydroxyquinoline sulphate (8-HQS) at 100, 200 and 300 ppm and sucrose at 5 or 10%, as well as all combinations between them. The flowers were placed in glass vials containing 400 ml holding solution of each treatment during the whole period of the experiment. The control flowers were treated only with distilled water. Three replications of six flowers each were used per treatment in these experiments and the cut flowers were arranged in a complete randomized design with the following treatments:

1. Control (Distilled water)
2. 100 ppm 8-HQS
3. 200 ppm 8-HQS
4. 300 ppm 8-HQS
5. 5% sucrose (5 g l^{-1})
6. 100 ppm 8-HQS + 5% sucrose
7. 200 ppm 8-HQS + 5% sucrose
8. 300 ppm 8-HQS + 5% sucrose
9. 10% sucrose (10 g l^{-1})
10. 100 ppm 8-HQS + 10% sucrose
11. 200 ppm 8-HQS + 10% sucrose
12. 300 ppm 8-HQS + 10% sucrose

Vase life determination

The longevity of *Strelitzia reginae* cut flowers was determined under laboratory conditions with normal daylight at 22±1°C and 60–70% RH and the number of open florets and vase life were evaluated daily. Flowers were discarded when the oldest fully opened floret was completely wilted and brown, as described by Finger et al. (1999). The floret longevity and vase life of *Hippeastrum vittatum* cut flowers were evaluated daily. The opened flowers were considered to be at the end of their vase life when the edges of the florets curled upwards and began to wilt (El-Saka et al., 2002) under laboratory conditions (26±1°C and 65–75% RH).

Fresh weight measurements

Fresh weight determinations of *Strelitzia reginae* and *Hippeastrum vittatum* cut flowers were made just before the immersion of the flowers in the solutions and were repeated on day 8, when the vase life of the control flowers was terminated at the stage mentioned above. The spikes were taken out of the solutions for as short a time as possible (20–30 s). The fresh weight of each flower was expressed relative to the initial weight, to represent the % weight increment for all treatments.

Soluble carbohydrate determination

The soluble carbohydrate concentrations were determined in both stems and florets of all treatments for the cut flowers tested in this study. Samples were taken on day 8 when the vase life of control flowers was terminated. The stem samples were taken from the middle of the stem and the floret samples from the second opening floret. The samples were oven dried at 70°C to constant weight and ground into a homogenized fine powder. A 0.5 g sub-sample of this powder was used for extracting the soluble carbohydrates. Total carbohydrates were determined according to Herbert et al. (1971) and calculated as mg g^{-1} dry weight.

Statistical analysis of results

The results were analysed using the SPSS program Base 9 (SPSS Inc., USA). The differences between means were evaluated using Duncan's multiple range test at the 0.05 level.

Results

Effect of 8-HQS and sucrose treatments on vase life

Strelitzia reginae

The data in Table 1 clearly indicate that all the 8-HQS and sucrose treatments and their combinations significantly extended both the vase life and the number of open florets of *Strelitzia reginae* cut flowers compared to the control. Generally, sucrose treatment was more effective than 8-HQS treatment. Combination of 8-HQS and sucrose resulted in the highest values in comparison with treatment with each individually. However, there was no significant difference between the 200 and 300 ppm 8-HQS treatments when sucrose was added at any level during the two experimental seasons.

The best results in this respect were obtained using 200 ppm 8-HQS + 10% sucrose treatment, when the vase life was 14.33 and 13.96 days and the number of florets opening 3.5 and 3.35 in the two seasons compared to 7.33 and 6.36 days and 1.86 and 1.76 florets for the control (Table 1).

Hippeastrum vittatum

Both the vase life and floret longevity of *Hippeastrum vittatum* cut flowers were prolonged as a result of using different 8-HQS and sucrose treatments during the two experimental seasons. The positive effect of 8-HQS was more apparent when combined with sucrose treatment. Treatment with 200 ppm 8-HQS + 10% sucrose resulted in maximum vase life (13.96 and 13.66 days) and floret longevity (7.66 and 7.86 days) in the first and second seasons, respectively (Table 2). The untreated control gave the lowest values in this respect (6.36 and 6.66 days for vase life and 4.11 and 4.13 days for floret longevity) during the two seasons.

Table 1

Effect of 8-hydroxyquinoline sulphate and sucrose treatments on the vase life (days) and number of florets opening for *Strelitzia reginae* cut flowers during the 2006 and 2007 seasons

Treatments	First season 2006		Second season 2007	
	Vase life	No. of florets opening	Vase life	No. of florets opening
Control	7.33i	1.86g	6.36j	1.76h
100 ppm 8-HQS	9.33f	2.33e	7.88h	2.20g
200 ppm 8-HQS	10.88d	2.62d	10.36e	2.68d
300 ppm 8-HQS	10.96d	2.63d	10.88d	2.72c
5% sucrose	8.66h	2.11f	7.44i	2.20g
10% sucrose	10.22e	2.51d	9.33f	2.40f
100 ppm 8-HQS + 5% sucrose	8.86g	2.15f	8.86g	2.20g
200 ppm 8-HQS + 5% sucrose	11.66b	2.62d	10.88d	2.56e
300 ppm 8-HQS + 5% sucrose	11.33c	2.63d	10.96c	2.55e
100 ppm 8-HQS + 10% sucrose	11.66b	2.81c	11.66b	2.88b
200 ppm 8-HQS + 10% sucrose	14.33a	3.52a	13.96a	3.35a
300 ppm 8-HQS + 10% sucrose	14.33a	3.33b	13.86a	3.33a

Means followed by different letters differ significantly from each other according to Duncan's multiple range test at P = 0.05.

Table 2
Effect of 8-hydroxyquinoline sulphate and sucrose treatments on the vase life (days) and floret longevity of *Hippeastrum vittatum* cut flowers during the 2006 and 2007 seasons

Treatments	First season 2006		Second season 2007	
	Vase life	Floret longevity	Vase life	Floret longevity
Control	6.36i	4.11h	6.66i	4.13f
100 ppm 8-HQS	7.88g	4.66g	8.11g	4.60e
200 ppm 8-HQS	10.36d	5.33f	10.66e	5.66d
300 ppm 8-HQS	10.88c	5.60e	10.96d	5.63d
5% sucrose	7.44h	4.66g	7.76h	4.33f
10% sucrose	8.33f	4.88g	8.66f	4.66e
100 ppm 8-HQS + 5% sucrose	8.86e	5.33f	8.33g	5.66d
200 ppm 8-HQS + 5% sucrose	10.88c	5.88d	11.00d	5.80d
300 ppm 8-HQS + 5% sucrose	10.96c	5.94c	11.13d	6.11c
100 ppm 8-HQS + 10% sucrose	11.66b	6.66b	12.00c	6.76b
200 ppm 8-HQS + 10% sucrose	13.96a	7.66a	13.66a	7.86a
300 ppm 8-HQS + 10% sucrose	13.86a	7.63a	13.33b	7.66a

Means followed by different letters differ significantly from each other according to Duncan's multiple range test at P = 0.05.

Effect of 8-HQS and sucrose treatments on the fresh weight

Strelitzia reginae

The effects of 8-HQS and sucrose treatments and their combination on the fresh weight of *Strelitzia reginae* cut flowers are summarized in Table 3. All the treatments applied significantly increased the percentage fresh weight gain at day 8, when the vase life of control flowers was terminated. In this respect, 8-HQS was more effective than sucrose treatment. However, when sucrose was combined with 8-HQS the percentage increment was significantly enhanced. The best treatment in this respect was 200 ppm 8-HQS + 10% sucrose, with values of 15 and 14.4% in the two seasons, compared to 2.5 and 2.4% for the control (Table 3).

Hippeastrum vittatum

The results in Table 4 clearly show that the percentage fresh weight gain from the initial weight of *Hippeastrum vittatum* cut flowers at day 8 was increased when 8-HQS and sucrose treatments were applied. The combined effect of 8-HQS and sucrose was better than the individual effects. The highest fresh weight gain was obtained using 200 ppm 8-HQS + 10% sucrose treatment in both seasons. This treatment increased the fresh weight by 11.5 and 10.6% in the two seasons compared to 2.9 and 1.9% in the control.

Table 3

Effect of 8-hydroxyquinoline sulphate and sucrose treatments on the fresh weight (g) and fresh weight gain (%) at the end of vase life for *Strelitzia reginae* cut flowers during the 2006 and 2007 seasons

Treatments	First season 2006			Second season 2007		
	Day 0	Day 8	Gain (%)	Day 0	Day 8	Gain (%)
Control	80	82	2.5 k	82	84	2.4 j
100 ppm 8-HQS	83	90	8.4 h	81	87	7.4 g
200 ppm 8-HQS	81	99	9.8 g	83	91	9.5 e
300 ppm 8-HQS	84	93	10.7 f	84	92	9.5 g
5% sucrose	82	86	4.8 j	80	84	5.0 i
10% sucrose	80	85	6.2 i	79	83	5.0 h
100 ppm 8-HQS + 5% sucrose	80	90	12.5 d	83	92	10.8 f
200 ppm 8-HQS + 5% sucrose	82	93	13.4 c	80	91	13.7 e
300 ppm 8-HQS + 5% sucrose	84	94	11.9 e	81	90	11.1 c
100 ppm 8-HQS + 10% sucrose	83	92	10.8 f	84	92	9.5 d
200 ppm 8-HQS + 10% sucrose	80	94	15.0 a	83	95	14.4 a
300 ppm 8-HQS + 10% sucrose	81	93	14.8 a	82	93	13.4 b

Means followed by different letters differ significantly from each other according to Duncan's multiple range test at P = 0.05.

Table 4

Effect of 8-hydroxyquinoline sulphate and sucrose treatments on the fresh weight (g) and fresh weight gain (%) at the end of vase life for *Hippeastrum vittatum* cut flowers during the 2006 and 2007 seasons

Treatments	First season 2006			Second season 2007		
	Day 0	Day 8	Gain (%)	Day 0	Day 8	Gain (%)
Control	101	104	2.9 i	102	104	1.9 j
100 ppm 8-HQS	103	107	3.8 h	103	108	4.8 g
200 ppm 8-HQS	102	108	5.8 f	102	109	6.8 e
300 ppm 8-HQS	104	109	4.8 g	101	106	4.9 g
5% sucrose	102	106	3.9 h	102	105	2.9 i
10% sucrose	102	107	4.9 g	101	105	3.9 h
100 ppm 8-HQS + 5% sucrose	101	107	5.9 f	103	109	5.8 f
200 ppm 8-HQS + 5% sucrose	105	112	6.6 e	102	109	6.8 e
300 ppm 8-HQS + 5% sucrose	103	111	7.7 d	103	112	8.7 c
100 ppm 8-HQS + 10% sucrose	105	114	8.5 c	101	109	7.9 d
200 ppm 8-HQS + 10% sucrose	104	116	11.5 a	103	114	10.6 a
300 ppm 8-HQS + 10% sucrose	103	114	10.6 b	102	112	9.8 b

Means followed by different letters differ significantly from each other according to Duncan's multiple range test at P = 0.05.

Effect of 8-HQS and sucrose treatments on the carbohydrate contents of the stem and florets

Strelitzia reginae

The carbohydrate content in the stems and florets of *Strelitzia reginae* cut flowers was positively affected by various 8-HQS and sucrose treatments (Table 5). Increasing 8-HQS or sucrose levels gradually increased the carbohydrate content in both stems and florets. All the treatments significantly increased the

carbohydrate content in both stems and florets in comparison with the control in both seasons, the highest values being 22.66 and 21.77 mg g^{-1} dry weight in the stems and 26.33 and 25.55 mg g^{-1} dry weight in the florets in the 200 ppm 8-HQS + 10% sucrose treatment in the two seasons, respectively. The control treatment resulted in the lowest values in this respect in both seasons (Table 5).

Hippeastrum vittatum

The data presented in Table 6 clearly show that all the 8-HQS and sucrose treatments significantly increased the carbohydrate content in both the stems and florets of *Hippeastrum vittatum* cut flowers in the two experimental seasons. In general, 8-HQS treatment was more effective than sucrose treatment. However, when sucrose was combined with 8-HQS the positive effect on the carbohydrate content was more pronounced. The best treatment in this respect was 200 ppm 8-HQS + 10% sucrose, which resulted in 23.96 and 22.86 mg g^{-1} dry weight for the stems and 24.18 and 23.75 mg g^{-1} dry weight for the florets in the two seasons, respectively, while the control treatment gave the lowest carbohydrate contents (10.33 and 9.88 mg g^{-1} dry weight in the stems and 11.53 and 10.15 mg g^{-1} dry weight in the florets) in the two seasons, respectively (Table 6).

Discussion

The results show the significance of 8-HQS and sucrose treatments in improving both the vase life and post-harvest quality of both types of cut flowers studied. These results could be explained through the role of 8-HQS as an anti-microbial agent. 8-HQS may have prevented the accumulation of microorganisms in the xylem vessels and suppressed xylem occlusion. The blockage of xylem vessels leads to water stress, which is well known to be the limiting factor of vase life, expressed in the form of early flower wilting (Henriette and Clerkx, 2001).

Table 5

Effect of 8-hydroxyquinoline sulphate and sucrose treatments on the carbohydrate content (mg g^{-1} dry weight) of stems and florets of *Strelitzia reginae* cut flowers during the 2006 and 2007 seasons

Treatments	First season 2006		Second season 2007	
	Stems	Florets	Stems	Florets
Control	12.33 h	13.86 h	11.88 g	13.33 h
100 ppm 8-HQS	16.55 f	19.11 e	16.33 j	18.88 e
200 ppm 8-HQS	18.77 d	20.88 d	17.88 f	19.76 d
300 ppm 8-HQS	17.88 e	21.11 cd	17.66 f	20.66 cd
5% sucrose	14.66 g	16.33 g	14.11 i	15.66 g
10% sucrose	16.33 f	18.23 f	15.88 h	18.33 f
100 ppm 8-HQS + 5% sucrose	18.66 d	21.66 c	18.33 e	21.33 c
200 ppm 8-HQS + 5% sucrose	20.33 b	23.66 b	19.88 d	23.11 b
300 ppm 8-HQS + 5% sucrose	20.66 b	23.33 b	20.11 c	22.77 b
100 ppm 8-HQS + 10% sucrose	19.33 c	23.66 b	19.66 d	23.33 b
200 ppm 8-HQS + 10% sucrose	22.66 a	26.33 a	21.77 a	25.55 a
300 ppm 8-HQS + 10% sucrose	22.33 a	25.88 a	21.16 b	24.96 a

Means followed by different letters differ significantly from each other according to Duncan's multiple range test at P = 0.05.

Table 6

Effect of 8-hydroxyquinoline sulphate and sucrose treatments on the carbohydrate content (mg g^{-1} dry weight) of stems and florets of *Hippeastrum vittatum* cut flowers during the 2006 and 2007 seasons

Treatments	First season 2006		Second season 2007	
	Stems	Florets	Stems	Florets
Control	10.33 i	11.53 i	9.88 h	10.15 h
100 ppm 8-HQS	14.46 g	15.66 g	13.66 ef	13.45 f
200 ppm 8-HQS	16.76 e	17.44 e	14.11 e	15.33 e
300 ppm 8-HQS	15.46 f	16.33 f	14.77 e	15.66 e
5% sucrose	12.15 h	13.33 h	11.33 g	11.88 g
10% sucrose	14.66 g	15.76 g	13.55 f	13.66 f
100 ppm 8-HQS + 5% sucrose	15.77 f	16.66 ef	15.66 d	15.88 e
200 ppm 8-HQS + 5% sucrose	17.88 d	18.55 d	16.42 d	17.56 d
300 ppm 8-HQS + 5% sucrose	19.44 c	20.45 c	17.77 c	19.42 c
100 ppm 8-HQS + 10% sucrose	21.87 b	22.16 b	19.33 b	21.33 b
200 ppm 8-HQS + 10% sucrose	23.96 a	24.18 a	22.86 a	23.75 a
300 ppm 8-HQS + 10% sucrose	23.66 a	24.22 a	22.45 a	23.15 a

Means followed by different letters differ significantly from each other according to Duncan's multiple range test at P = 0.05.

It is clear from the results that 8-HQS treatment led to the maximum fresh weight gain in the cut flowers. The increment in fresh weight may be due to its additional role in increasing water uptake (Hassan et al., 2003). However, there was limited water uptake in untreated flowers and the unbalance between water uptake and transpiration finally led to irreversible damage and the premature end of vase life (Van Meeteren et al., 2001). This explains the short vase life of the untreated control and the long vase life when 8-HQS was applied, because the transport of water and minerals is of vital importance for flower development. The obstruction of the xylem vessels is a commonly occurring problem in cut flowers. These results are in harmony with the findings of El-Saka et al. (2002), Kim and Lee (2002) and Reddy et al. (1995). The effectiveness of 8-HQS in improving the post-harvest quality of *Strelitzia reginae* and *Hippeastrum vittatum* cut flowers may also be due to its role in increasing the carbohydrate contents of both stems and florets, as shown in Tables 5 and 6, which may be reflected in an increase in the vase life and quality of cut flowers. The low carbohydrate content of untreated flowers resulted in the lowest vase life in both seasons.

Sucrose treatment led to increases in the fresh weight and carbohydrate content of stems and florets, thus enhancing the post-harvest quality of *Strelitzia reginae* and *Hippeastrum vittatum* cut flowers. The positive effect of sucrose treatment could be explained by its role as a source of nutrition for tissues approaching carbohydrate starvation. It may also act as an osmotically active molecule, influencing flower opening, subsequent water relations and the maintenance of flower turgidity. These results are in agreement with the findings

of Abdel-Kader et al. (2004), who reported that the higher the content of reducing sugars in the petals, the longer the vase life of dahlia flowers. The results also indicated a significant interaction between 8-HQS and sucrose treatment, which maximized water uptake and maintained the water balance, resulting in a high rate of photosynthesis and an increase in the carbohydrate percentage, thus prolonging the vase life of cut flowers. These results are in agreement with the findings of Kim and Lee (2002), O'Donoghue et al. (2002) and Hassan et al. (2008).

It could be concluded from the results that treatment with 200 ppm 8-HQS + 10% sucrose could be recommended to obtain the highest post-harvest quality of *Strelitzia reginae* Ait. and *Hippeastrum vittatum* Herb. cv. Apple Blossome cut flowers.

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COMBINING ABILITY STUDY FOR GRAIN YIELD AND YIELD-RELATED TRAITS OF GRAIN SORGHUM [*Sorghum bicolor* (L.) Moench] IN ETHIOPIA

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A field experiment was conducted in three low moisture areas in Ethiopia to study general and specific combining ability (GCA and SCA) for developmental, panicle and grain traits. Twenty-four parents (6 male sterile lines and 18 fertility restorers or testers) were used along with their 108 crosses. Combining ability analysis through a line × tester set for 14 agronomic traits in three environments and on a pooled basis revealed the importance of both additive and non-additive genetic effects. However, the predominance of additive components was observed in the inheritance of most traits studied. Environments also influenced the GCA and SCA effects. The female genotypes ICSA-10, ICSA-15 and ICSA-30, the testers ICSR-14 and KLCTENT # 17DTN and the crosses ICSA-30 × KCTENT # 17DTN, ICSA-30 × ICSR-16, ICSA-15 × KCTENT #17DTN and ICSA-10 × ICSR-14 exhibited the highest and significant SCA effects for yield and some of its components, indicating their importance in hybrid development for lowland areas of Ethiopia.

Key words: agronomic traits, combining ability, line × tester, *Sorghum bicolor*

Introduction

Traditionally, in lowland areas of Ethiopia, where moisture is the limiting factor, sorghum [*Sorghum bicolor* (L.) Moench] is one of the most important cereal crops. It is planted as food insurance, especially in the lowlands of Eastern Ethiopia, such as the Mieso-Asebot plains, in the Ogaden region, and in the north and north-eastern parts of the country. The grain is primarily used as food, whereas the stalks are used for animal feed, fuel and construction material. The climate of these areas is characterized by unpredictable drought and erratic rainfall. Also, the growing season is short. Thus, the selection of early maturing varieties/hybrids and the choice of appropriate parents is of paramount importance, requiring the testing of the parents for combining ability. The breeding value of any material is largely determined by its combining ability for important productivity-related traits (Harer and Bapat, 1982; Pathak and Sanghi,

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1992). Furthermore, the knowledge of gene action is of great value, as it varies depending on the genetic variation and genetic divergence in the material. Combining ability studies provide useful information for the selection of parents in terms of the performance of the hybrids, and elucidate the nature and magnitude of various types of gene actions involved in the expression of quantitative traits (Goyal and Kumar, 1991). Information on these aspects is inadequate in lowland grain sorghum under Ethiopian conditions. Keeping this in view, some of the cytoplasmic male sterile lines introduced from ICRISAT were crossed with genetically and geographically diverse restorers having better agronomic attributes and acceptable grain quality, to study the general and specific combining ability (GCA and SCA) for developmental, panicle and grain traits.

Materials and methods

Six male sterile lines were crossed with 18 fertility restorers (Table 1) to produce the seed of F_1 hybrids. A hundred and eight crosses along with the 24 parents were grown in a randomized block design with three replications at three locations, namely Melkassa, Mieso and Babile research sites, during the 1998 crop season (Table 2). Each genotype was planted in a one-row plot three metres in length. The spacing was 75 cm between rows and 15 cm between plants. Each plot was used for the evaluation of yield and its components. The normal cultural practices recommended for the production of sorghum were applied to all the plots.

Table 1
Identification of the male and female parents used in combining ability studies at Babile, Melkassa and Mieso, Ethiopia in 1998

No.	Identification	Type of parent	Origin*
1	ICSA-34	Line (female)	ICRISAT, India
2	ICSA-5	Line (female)	ICRISAT, India
3	ICSA-10	Line (female)	ICRISAT, India
4	ICSA-21	Line (female)	ICRISAT, India
5	ICSA-30	Line (female)	ICRISAT, India
6	ICSA-15	Line (female)	ICRISAT, India
7	ICSR-14	Tester (male)	ICRISAT, India
8	ICSR-16	Tester (male)	ICRISAT, India
9	ICSR-50	Tester (male)	ICRISAT, India
10	ICSR-117	Tester (male)	ICRISAT, India
11	ICSR-113	Tester (male)	ICRISAT, India
12	ICSR-161	Tester (male)	ICRISAT, India
13	P89001	Tester (male)	Purdue University, USA
14	P89002	Tester (male)	Purdue University, USA
15	P89006	Tester (male)	Purdue University, USA
16	P89008	Tester (male)	Purdue University, USA
17	P-46-1	Tester (male)	EARSAM
18	MR747	Tester (male)	EARSAM
19	SK-82-022	Tester (male)	EARSAM
20	91MK7013	Tester (male)	Ethiopia (from crossing programme)
21	90MW5344	Tester (male)	Ethiopia (from crossing programme)
22	82LPYT-2#5 x 81ESIP 46	Tester (male)	Ethiopia (from crossing programme)
23	KCTENT # 17DTN	Tester (male)	EARSAM
24	Meko-1	Tester (male)	EARSAM

*ICRISAT: International Crops Research Institute for Semi Arid Tropics; EARSAM: East Africa Regional Sorghum and Millets Improvement Network

Table 2
Description of the testing environments for the line × tester study on lowland grain sorghum, 1998

Location	Altitude (m.a.s.l)	Latitude	Longitude	Soil type
Melkassa	1550	8°26'N	39°22'E	Sandy loam
Mieso	1420	9°13'N	40°46'E	Clay loam
Babile	1650	9°12'N	42°18'E	Sandy loam

Data were recorded on the following agronomic traits:

- Seedling vigour: Visual scoring of the seedlings between 10 and 20 days after emergence using a 1–5 scale, where 1 is the most vigorous and 5 is the least vigorous seedling in the plot (House, 1985).
- Days to 50% flowering: Number of days from planting till 50% of the plants in a plot showed flowering halfway down the panicle.
- Number of leaves per plant: Recorded on five random plants per plot and averaged.
- Number of primary branches per panicle: The average number of primary branches on five random plants.
- Plant height (cm): Height from the ground to the panicle tip of five random plants per plot.
- Number of kernels per panicle: Recorded as the average of five random panicles per plot.
- Panicle weight (g): Average weight of five random panicles per plot.
- Grain yield (g): Weight of threshed grains per plot.
- 100-kernel weight (g): Recorded for 100 seeds from each panicle used for panicle weight, at 12.5% moisture content, and averaged.
- Panicle length: Distance in cm from the panicle tip to the lowest panicle branch on five random plants per plot.
- Threshing percentage: WG ×100/WP, where: WG = weight of threshed panicles; WP = weight of panicles before threshing
- Leaf length (cm): Average length of the fourth leaf from the flag leaf on five random plants per plot.
- Leaf width (cm): Average width of the fourth leaf from the flag leaf at the widest point on five random plants per plot.
- Leaf area: Expressed as length × width of the fourth leaf from the top × 0.747

Data analysis

Analysis of variance for a randomized complete block design was carried out for all the traits. For traits where significant differences between the crosses were observed, the data were analysed using line × tester analysis, as described by Kempthorne (1957). All traits were analysed separately for each environment as well as for pooled data over locations. The statistical analysis was carried out using GenStat software, and the significance of combining ability estimates was analysed using the t-test.

Results and discussion

Environment had a significant effect on the expression of all 14 traits (Table 3). Mean squares due to hybrids were also significant for all traits, suggesting that further genetic analysis on combining ability could give more information on the GCA of the lines (females) and testers (males).

Table 3
Mean squares in combined analysis of variance across three environments for yield and yield component traits at Babile, Melkassa and Mieso, Ethiopia in 1998

Source	Df	Seedling vigour	Days to 50% flowering	Leaf number per plant	Leaf length (cm)	No. of primary branches	Plant height (cm)	No. of seeds per plant
E	2	30.153	8848.38**	398.9112**	49081.98**	30243.2**	480285.8**	41200000**
P	23	2.017*	98.04**	4.5947**	188.36**	135.13**	2583.3**	1864000**
P × H	1	5.882*	2359.49**	0.1167	656.6**	1498.57**	66291.7**	25050000**
E × P × H	2	16.946	155.46**	3.5047*	178.35**	221.66*	2173.0**	73777000**
T	17	2.291*	96.07**	2.6434**	308.13**	138.92**	7978.1**	3655000**
L	5	10.201**	293.05**	2.4150*	863.46**	1135.84**	6462.3**	1150000**
E × P	46	1.312	25.65	1.4960**	83.21**	91.59**	409.1**	455800**
E × T	34	1.438	29.87	1.7764**	59.79**	91.62**	604.6**	464900**
E × L	10	4.747*	128.59**	2.9845**	168.6**	343.5**	505.0*	1589000**
L × T	84	1.198	47.97**	1.3592**	64.38**	53.00	391.1**	470900**
E × L × T	166	1.450*	45.12**	0.157	47.35**	56.07	313.9**	339800*
Pooled error	773	0.5197	2.493	0.443	2.684	3.478	7.290	250.4**

Source	Df	Panicle weight (g)	Grain yield (g)	100-kernel weight (g)	Panicle length (cm)	Threshing percentage	Leaf width (cm)	Leaf area
E	2	56345.2**	43420000**	4.9778*	2435.1*	8211.52**	551.7053**	5212114**
P	23	1462.4**	70340**	1.1752**	57.7	22057**	5.0920**	29896**
P × H	1	44062.3**	1921000**	3.5302**	2675.4**	488.00**	13.0902**	121297**
E × P × H	2	19641.3**	506500**	1.9781**	242.4	3.96	3.6436*	36913**
T	17	6946.0**	238300**	1.7825**	271.4*	418.74**	5.5420**	45951**
L	5	3140.3**	191500**	2.0321**	309.6**	171.99**	14.0086**	93762**
E × P	46	579.7	23020*	0.2200*	18.3	8646**	2.0988**	13788**
E × T	34	1270.3*	58300**	0.3300**	184.9	98.25**	1.4997**	9260**
E × L	10	3065.7**	150200**	0.4913**	145.0	70.44**	2.4236**	12157**
L × T	84	1403.5**	27770**	0.1596	212.4*	37.17	1.0087	6925**
E × L × T	166	1301.5**	20980*	0.1536	197.6*	41.98	1.0614*	6161**
Pooled error	773	14.12	61.63	0.1790	6.016	2.840	0.4457	32.36

E: Environment; P: Parents; H: Hybrid; T: Testers (male); L: Lines (female); *, **: Significant at $P<0.05$ and $P<0.01$, respectively

The mean squares due to general combining ability (GCA) and specific combining ability (SCA) were highly significant in the pooled environment for most traits studied, indicating that both the additive and non-additive components of genetic variance controll these traits (Table 3). Mean squares due to GCA (testers) × environments and GCA (lines) × environments were also highly significant for all traits except for seedling vigour, days to 50% flowering and panicle length. Similar results were reported by Singhania (1980) and Toure et al. (1996) for plant height, panicle length, number of seeds per panicle, 100-kernel weight and grain yield, and by Shah and Joshi (1996) for plant height. Yadav et al. (2000) obtained similar results in pearl millet for grain yield and 100-kernel weight, and Shewangizaw et al. (1985) for grain yield in maize. The GCA component was larger than that of SCA in all traits studied. This finding

was in agreement with earlier sorghum data (Niehaus and Pickett, 1966) for grain yield, number of seeds per panicle, threshing percentage, days to 50% flowering, number of leaves per plant and plant height. Also, SCA variances due to the female × male interaction were found for all traits except for seedling vigour, number of primary branches per panicle, 100-kernel weight, threshing percentage and leaf width. This confirmed the results of other authors (Goud et al., 1973; Harer and Bapat, 1982) for days to 50% flowering, leaf area, panicle weight, grain yield and leaf width. Furthermore, significant combining ability variances due to the environments × SCA interaction, i.e. E × Lines × Testers (Table 3) were found for all traits except for leaf number per plant, number of primary branches, 100-kernel weight and threshing percentage. This result is in agreement with the results obtained by Toure et al. (1996) for grain yield, number of seeds per panicle, plant height, days to 50% flowering and panicle length. Since the GCA and SCA mean squares revealed significance at the $P<0.01$ level for grain yield, it is suggested that both additive and non-additive variances were important in grain response. This result is supported by the findings of Toure et al. (1996), Rao (1970; 1972) and Nagur and Murty (1970), which showed that additive as well as non-additive gene action controlled the inheritance of grain yield and its components because, genetically, GCA is associated with genes that are additive in their effects, while SCA is attributed primarily to deviation from the additive scheme caused by dominance and epistasis (Kambal and Webster, 1965). In the present study the variance due to GCA was prominent for all the traits. Similar results were found by Harer and Bapat (1982), Toure et al. (1996) and Shankaregowda et al. (1972) for plant height, number of leaves per plant, leaf length, leaf area, days to 50% flowering, panicle length and width and 100-kernel weight, indicating the presence of additive gene effects for these traits. The larger σ^2 GCA for grain yield and number of seeds per panicle provides the genetic conditions that could profitably be employed in hybrid programmes by incorporating diverse parents. This is in conformation with the results of Goud et al. (1973) and Bapat (1973).

The percentage of sum of squares for crosses due to GCA and SCA across environments (Table 4) indicated that total GCA (GCA testers plus GCA lines) accounted for most of the variability between crosses for all traits except for days to 50% flowering, panicle length, seedling vigour and number of leaves per plant. The parental GCA accounted for 68.23%, 75.11%, 63.18%, 83.64% and 71.88% of the total variability between crosses for grain yield, 100-kernel weight, number of seeds per panicle, plant height and threshing percentage, respectively. GCA testers accounted for the highest percentage (75%) contribution in parents for 100-kernel weight, number of seeds per panicle, plant height and threshing percentage, and GCA lines for 55.19% of the total variability for grain yield. GCA was greater than SCA, suggesting that both additive and non-additive effects are important in grain yield determination, but with a preponderance of low non-additive effects. A similar trend was observed

by Toure et al. (1996), Malm (1968), Harer and Bapat (1982) and De Franca (1990). In all three environments, total GCA accounted for a lower percentage of the variability between crosses for seedling vigour (Table 4). Thus, non-additive variance was more important than additive variance in determining seedling vigour for these crosses.

The interaction of environment with lines and testers was significant for all traits except for seedling vigour and days to 50% flowering, showing the environmental sensitivity of GCA effects and emphasizing the need for appropriately targeting genotypes for specific environments (Table 3). Moreover, the significant interactions of genetic effects with environment within certain agro-ecological regions emphasize the need for testing at more than one site within the target region for a precise estimate of GCA effects. The SCA effects controlling all traits except leaf number per plant, number of primary branches, 100-kernel weight and threshing percentage were also influenced by environment, as indicated by the significant interaction of line × tester with environment. The occurrence of environmental interaction with genotypic effects has been reported in several studies in sorghum and pearl millet (Yadav et al., 2000; Toure et al., 1996; Kambal and Webster, 1965; Lodhi et al., 1977;1978; Saini and Paroda, 1977; Jagadeshwar and Shinde, 1992; Shah and Joshi, 1996). This implied that the estimates obtained in a single environment are potentially biased. In the present investigation, the three environments allowed an unbiased estimate of combining ability effects to be obtained.

Table 4

Percentage of the sum of squares attributable to general and specific combining ability for fourteen traits pooled over environments (at Babile, Melkassa and Mieso, Ethiopia in 1998)

Traits	GCA Testers (Male)	GCA Lines (Female)	SCA (Line × Tester)
Grain yield	13.04	55.19	31.77
100-kernel weight	56.25	18.86	24.89
No. of primary branches per panicle	18.90	45.46	35.64
No. of seeds per panicle	57.83	5.35	36.82
Days to 50% flowering	22.91	20.56	56.53
Plant height	67.55	16.09	16.36
Panicle length	19.22	6.45	74.33
Leaf width	37.84	28.13	34.03
Panicle weight	46.92	6.24	46.84
Threshing percentage	64.13	7.75	28.13
Leaf area	42.65	25.59	31.76
Seedling vigour	20.44	26.77	52.79
Leaf number per plant	26.25	7.05	66.70
Leaf length	35.01	28.85	36.14

GCA: General combining ability; SCA: specific combining ability

The estimates of GCA effects for parents are presented in Table 5. It is obvious that the female parents ICSA-10, ICSA-15 and ICSA-30 (in that order) appeared to be good general combiners for grain yield. The lines ICSA-10 and ICSA-15 had highly significant positive GCA effects for panicle weight, while line ICSA-10 also had significant positive GCA effects for panicle length, threshing percentage, leaf length, number of primary branches per panicle, days to 50% flowering and seedling vigour. Thus, this parent was found to be the best general combiner for these traits. The lines ICSA-30 and ICSA-15 can be used in producing high yielding combinations with medium height. Similarly, lines ICSA-5 and ICSA-21 were the best combiners for height. The line ICSA-10 could be used successfully in hybridization programmes to produce dwarf, vigorous seedlings and good yielding hybrids, as is evident from its GCA effect.

Table 5a

Estimates of GCA effects of lines and testers for grain yield and its components in lowland grain sorghum pooled over environments (at Babile, Melkassa and Mieso, Ethiopia in 1998)

Parent	Grain yield (g/plot)	100-kernel weight (g)	No. of seeds per panicle	Panicle weight (g)	Panicle length (cm)	Threshing percentage	Leaf width (cm)
Lines (female)							
ICSA-34	–3.86	–0.226	122.76*	0.817	–0.286	0.8560	0.40085*
ICSA-5	–40.16*	0.0615	–49.19	1.164	1.696	–0.7352	0.2663*
ICSA-10	41.11*	–0.1879*	–28.71	6.334*	2.140*	1.6765*	–0.1094
ICSA-21	20.38	0.0466	–98.35*	3.146	1.277	0.8066	–0.3922*
ICSA-30	25.96*	0.1398*	–28.49	0.854	–0.619	0.7573	–0.1699*
ICSA-15	38.78*	–0.0374	82.48	6.644*	0.072	–0.0082	–0.0033
SE	10.27	0.0298	41.74	2.353	1.003	0.4733	0.0743
Testers (male)							
ICSR-14	107.47*	0.2413*	51.6	10398*	0.154	0.491	0.2154
ICSR-16	65.01*	0.0509	248.6*	9.755	5.270*	0.25	0.2992*
ICSR-50	45.99*	0.1490*	187.9*	11.700	0.363	1.084	0.3362*
ICSR-117	–11.24	–0.1658*	287.8*	0.978	–1.175	–0.360	0.0122
ICSR-133	26.65	–0.1098*	377.6*	4.144	0.381	0.809	0.2899*
ICSR-161	39.76*	–0.1343*	283.9*	21.681*	0.344	–1.045	0.5622*
P89001	–60.51*	–0.1880*	–95.8	–11.115*	–1.471	1.529	–0.2934*
P89002	–33.22	–0.0973	–204.6*	–12.078*	5.196*	–1.453	–0.0989
P89006	–120.62*	–0.3065*	–261.0*	–15.559*	0.399	–7.175*	–0.5249*
P89008	–1.27*	–0.1676*	–375.8*	–11.745*	0.122	–4.990*	–0.3304*
P-46-1	–105.62*	–0.2454*	–329.0*	–16.300*	0.788	–2.730*	–0.3489*
MR747	–1.29	0.2898*	–282.4*	1.144	–2.101	–1.008	0.1974
SK-82-022	48.93*	0.0731	33.8	–3.096	–2.527	2.696*	–0.0712
91MK 7013	–20.51	0.1639*	–206.1*	–6.856	–0.712	2.066*	–0.2080
90MW5344	–8.31	0.1139*	–144.9	–4.411	–0.804	1.196	–0.1915
82LPYT-2#5x 81ESIP 46	–3.59	0.0861	–6.2	–0.670	–2.804	3.770*	–0.3582*
KCTENT #17 DTN	102.12*	0.0083	479.3*	18.848*	0.883	2.696*	0.4936*
Meko-1	29.97	0.2385*	–44.6	3.181	–2.304	2.177*	0.0122
SE	17.79	0.0517	72.30	4.076	1.737	0.8198	0.1287

*: Significant at $P<0.05$

Table 5b
Estimates of GCA effects of lines and testers for yield and its components in lowland grain sorghum pooled over environments (at Babile, Melkassa and Mieso, Ethiopia in 1998)

Parent	Leaf length	Number of primary branches	Plant height	Days to 50% flowering	Leaf number per plan	Leaf area	Seedling vigor
Lines (female)							
ICSA-34	1.134*	–3.455*	–9.794*	1.364*	0.0590	24.93*	0.0181
ICSA-5	2.978*	2.689*	6.666*	0.540	0.1770*	29.48*	–0.1901*
ICSA-10	3.076*	3.210*	–5.730*	1.104*	0.0252	–27.68*	0.2931*
ICSA-21	0.712	0.340	3.027*	2.204*	0.0096	–22.59	0.3554*
ICSA-30	–0.416	–2.282*	2.700*	–0.673	–0.1694*	–9.74	0.2563*
ICSA-15	0.898	–0.510	3.132*	–0.050	–0.1015	5.60	–0.0220
SE	0.4474	0.5796	1.215	0.4155	0.0738	5.39	0.0866
Testers (male)							
ICSR-14	3.456*	1.760*	7.232*	0.959	0.3055*	33.26*	–0.0959
ICSR-16	2.041*	3.650*	2.304	2.395*	0.3306*	27.75*	0.0644
ICSR-50	1.948*	0.428	2.175	1.228	0.0899	27.14*	0.4903*
ICSR-117	0.652	3.0534*	2.638	0.895	0.0027	2.44	0.0745
ICSR-133	0.504	1.372	–5.362*	–0.105	–0.0212	17.09	–0282
ICSR-161	3.967	1.743	1.619	2.783*	0.3491*	53.05*	–0.0968
P89001	–2.570*	–0.905	–7.436*	–0.642	–0.1138	–29.65*	–0.0930
P89002	0.115	–1.683	–5.233*	–1.013	–0.2064	–5.41	0.0783
P89006	–1.792*	–1.220	–24.288*	0.191	–0.3731*	–35.64*	–0.1578
P89008	–4.589*	1.076	–10.047*	–1.476	0.21.95	–43.82*	0.0922
P-46-1	–3.385*	–1.776	–28.325*	–1.142	–0.2249	–37.21*	–0.0560
MR747	0.300	–0.220	3.379	0.006	0.0158	11.90	0.2070
SK-82-022	–0.292	0.928	9.583*	–2.013*	–0.0027	–7.54	–0.4912*
91MK 7013	–1.718*	–0.442	–1.325	–0.291	–0.1879	–20.65*	0.0883
90MW5344	–1.237	–0.942	15.212*	–1.235	–0.0768	–18.34	0.0459
82LPYT-2#5x 81ESIP 46	0.041	–0.650	11.453*	–0.013	–0.2064	–17.49	0.2866
KCTENT #17 DTN	3.356*	–0.479	10.175*	0.913	0.2936*	44.59*	–0.1486
Meko-1	–0.996	–0.887	16.249*	–1.439	–0.1879	–1.45	0.0459
SE	0.7749	1.004	2.105	0.7197	0.1279	–.342	0.1500

*: Significant at P<0.05

Among the testers (Table 5) ICSR-14 and KCTENT # 17 DTN were the best general combiners for yield, followed by SK-82-022, ICSR-50 and ICSR-161, while P89001, P89006, P89008 and P-46-1 were the worst combiners for yield. The tester P89006 showed significant negative GCA effects for all traits except panicle length, number of primary branches, days to 50% flowering, and seedling vigour. Other testers (ICSR-14, ICSR-16, ICSR-161 and P89008) showed significant negative GCA effects for more than 50% of the traits. For earliness and tallness, the tester SK-82-022 was the best general combiner and could thus be utilized in conventional breeding programmes to induce earliness and tallness. Among the female parents, ICSA-30 was a good general combiner for 100-kernel weight, whereas the male parents ICSR-14, ICSR-16, 91MK 7013, 90MW5344 and Meko-1 were better combiners for this trait, suggesting their suitability for producing large-seeded hybrids for lowland Ethiopian

conditions. In general, high combiners for grain yield in these materials also seemed to show high combining ability effects for one or more traits, such as panicle weight, 100-kernel weight, leaf length, leaf width and area, threshing percentage, number of seeds, number of primary branches per panicle, panicle length and seedling vigour.

Based on the present study, the lines ICSA-10, ICSA-15 and ICSA-30 and the testers ICSR-14, KCTENT # 17 DTN, ICSR-16 and ICSR-50 would be useful for developing new hybrid combinations for lowland areas of Ethiopia. Specific combining ability effects represent the dominance and epitasis components of variation, which are non-fixable in nature (Shah and Joshi, 1996). However, crosses with high SCA effects involve one or both parents that are good general combiners. Such materials could be successfully exploited in varietal improvement programmes. In the present investigation five F_1 hybrids with positive, highly significant SCA effects had the high × high GCA combination, while one hybrid (ICSA-15 × P-46-1) had the high × low combination for grain yield (Table 6). However, in a number of combinations, parents with high × low GCA for seedling vigour, leaf number per plant and leaf length, high × high for grain yield and high × medium for panicle length were also involved, indicating the role of dominance gene action in the expression of these traits. Generally, these cross combinations involved good, average and poor combiners. Thus, it could be concluded that both inter- and intra-allelic interactions were involved in the expression of these attributes. Harer and Bapat (1982) and Chauhan and Singh (1974) reported similar results. In summary, the SCA effects indicate non-additive gene action for the inheritance of yield and yield-contributing traits, revealing that hybrids with a high manifestation of vigour could be obtained. Furthermore, the superior parents identified could be gainfully used in breeding programmes to develop high-yielding materials for lowland grain sorghum in Ethiopia.

Table 6

Estimate of SCA effects of the five best crosses for grain yield and its components, pooled over environments (at Babile, Melkassa and Mieso, Ethiopia in 1998)

Crosses	Grain yield	100-kernel weight	No. of seeds per panicle	Panicle weight	Panicle length	Threshing percentage
ICSA-30 × KCTENT # 17 DTN	102.00**	0.0120	211.1*	8.553	2.101	0.002
ICSA-30 × ICSR-16	208.32**	–0.0152	824.7*	32.261*	1.508	4.650*
ICSA-15 × KCTENT # 17 DTN	101.72**	0.1967	–130.7	2.738	–4.733	0.798
ICSA-15 × P-46-1	94.13**	0.0491	–99.7	5.405	1.746	–0.720
ICSA-10 × ICSR-14	92.58**	–0.0811	105.4	12.072	1.212	–0.541
ICSA-30 × KCTENT # 17 DTN	0.2070	0.269	3.856	5.745	0.062	0.0528
ICSA-30 × ICSR-16	0.4837	7.282*	4.792	20.741*	–2.484	0.3045
ICSA-15 × KCTENT # 17 DTN	–0.2190	–2.843	–2.755	15.708*	–1.512	–0.4973
ICSA-15 × P-46-1	0.0773	0.916	1.726	3.115	–1.197	0.2435
ICSA-10 × ICSR-14	0.0082	0.959	–0.453	3.109	–0.679	0.3114

*,**: Significant at $P<0.05$ and $P<0.01$, respectively

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STABILITY ANALYSIS OF SEED YIELD IN SAFFLOWER GENOTYPES IN IRAN

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To assess the stability and yield performance of safflower genotypes and to identify subregions within Iran, a set of experiments was conducted at six locations during 2003–2005. AMMI model analysis and some stability parameters derived from the grain yield were used. AMMI analysis showed differences between genotypes and environments and the GE interaction was highly significant, indicating that the agro-climatic environmental conditions were different, and that there was a differential response of the genotypes to the environments. The first two IPCA components of the GE interaction explained 51.5% of the GE interaction. According to the AMMI model, G16 was the most superior genotype in 15 out of 18 environments. The biplot of IPCA1 and IPCA2 showed that the six locations represent different environments, and mega-environments in Iran were identified for safflower breeding programmes. Due to the great fluctuation observed when selecting genotypes through stability parameters, it was not possible to distinguish stable genotypes clearly. In addition, when calculating these parameters high yield performance is not considered. So the Yield and Stability Index (YSI) can be recommended as a new approach to facilitate genotype selection, where genotypes with low values of YSI are the best. According to YSI the genotypes G16, G2, G9 and G1 can be selected. These genotypes were also selected using the AMMI model.

Key words: AMMI analysis, safflower, stability parameters

Introduction

The first goal of plant breeders in a crop breeding programme is the development of cultivars or genotypes which are stable or adapted to a wide range of diversified environments (Arshad et al., 2003). A secondary, but important, goal is to develop an understanding of the target region and, in particular, to determine if the target region can be subdivided into different mega-environments. Multiple environment trials (MET) are conducted annually throughout the world by various breeding institutions and seed companies. These

are essential because of the presence of genotype × environment (GE) interactions, i.e. differential genotypic responses to different environments (Casanoves et al., 2005). The GE interaction complicates the identification of superior genotypes and needs to be modelled and interpreted. Models may be linear formulations such as joint regression (Yates and Cochran, 1938; Finlay and Wilkinson, 1963; Eberhart and Russell, 1966; Perkins and Jinks, 1968), factorial regression (Denis, 1980; 1988) or additive main effect and multiplicative interaction (AMMI) on MET data.

AMMI analysis has been shown to be more effective than the conventional two-way fixed effects model with interaction (Zobel et al., 1988), because it achieves several important goals including (i) parsimony, because the model contains relatively few of the interaction degrees of freedom, (ii) effectiveness, because the model contains most of the interaction sum of squares (SS), which is rich in pattern, leaving a residual that is rich in noise, with most of the degrees of freedom but small SS, thereby affording greater predictive accuracy and statistical efficiency (Gauch, 1992; Gauch and Zobel, 1996), and (iii) mega-environment analysis (Gauch and Zobel, 1997), which identifies homogeneous subregions within a crop's growing region having similar GE interactions. AMMI can simplify cultivar recommendations by reducing the number of winning genotypes through gains in statistical efficiency and accuracy (Gauch, 1988; 1990; 1992; Gauch and Zobel, 1988; 1996) and by combining multiple test site locations into regions having similar cultivar recommendations (Peterson and Pfeiffer, 1989; Gauch and Zobel, 1997). The objectives of this research were (i) to recommend genotypes based on stability parameters and the AMMI model, (ii) to identify subregions within the safflower growing region in Iran, and (iii) to study the relationships between the studied parameters.

Materials and methods

Data source

This study was carried out with 16 safflower genotypes (Table 1a) across 18 environments involving six dryland agricultural research stations, namely Sararood (Kermanshah province), Ardebil (Ardebil province), Ghamloo (Kordestan province), Gonbad (Golestan province), Shirvan (North Khorasan province) and Khoram-abad (Lorestan province) during three years, 2003–2005 (Table 1b). The experiments were conducted in a randomized complete block design (RCBD) with three replications in each environment. Sowing was done by hand in 1.5 m × 4 m plots, consisting of five rows with 30 cm row spacing. The seeding rate was 30 seeds m^{-2} for each location. Fertilizer was applied as 40 kg ha^{-1} nitrogen and 60 kg ha^{-1} P_2O_5 at planting and 40 kg ha^{-1} N as top dressing in early spring. Yield (kg ha^{-1}) was obtained by converting the grain yields obtained for each plot.

Statistical analysis

Similarities between test environments were evaluated based on environmental main effects and GE interaction effects using AMMI analysis, which is a combination of analysis of variance and multiplication effect analysis. Briefly, analysis of variance is used to partition variance into three components: genotype deviations from the grand mean, environment deviations

from the grand mean, and GE deviations from the grand mean. Subsequently, multiplication effect analysis is used to partition GE deviations into different interaction principal component axes (IPCA), which can be tested for statistical significance through ANOVA. The AMMI analysis is interpreted by plotting the IPCAs of GE in various types of biplots. The Genstat and IRRISTAT software was used for AMMI analysis.

Table 1a
Code, cropping season and rainfall status for each environment

Environment code	Cropping season	Station	Province	Precipitation (mm)
A	2002–03	Sararood	Kermanshah	424.4
B	2002–03	Ardebil	Ardebil	274.0
C	2002–03	Ghamloo	Kordestan	354.0
D	2002–03	Gonbad	Golestan	444.7
E	2002–03	Shirvan	North Khorasan	301.0
F	2002–03	Khoram-abad	Lorestan	335.4
G	2003–04	Sararood	Kermanshah	588.0
H	2003–04	Ardebil	Ardebil	282.0
I	2003–04	Ghamloo	Kordestan	425.0
J	2003–04	Gonbad	Golestan	492.8
K	2003–04	Shirvan	North Khorasan	251.0
L	2003–04	Khoram-abad	Lorestan	466.7
M	2004–05	Sararood	Kermanshah	431.5
N	2004–05	Ardebil	Ardebil	286.2
O	2004–05	Ghamloo	Kordestan	333.7
P	2004–05	Gonbad	Golestan	700.6
Q	2004–05	Shirvan	North Khorasan	242.2
R	2004–05	Khoram-abad	Lorestan	482.9

Table 1b
Names and origin of genotypes

Code	Genotype	Origin
G1	CH-5	America
G2	PI-250537	World Bank of Safflower
G3	Syrian	Syria
G4	CW-74	America
G5	Dincer	Turkey
G6	Zarghan279	Iran
G7	LRV-55-245	Iran
G8	PI-198290	World Bank of Safflower
G9	Hartman	America
G10	Gila	America
G11	Kino-76	ICARDA
G12	Yenice	Turkey
G13	PI-537636	World Bank of Safflower
G14	PI-537636-s	World Bank of Safflower
G15	LRV-51-51	Iran
G16	PI-537598	World Bank of Safflower

To evaluate genotype stability and to further study GE, stability analysis was applied to the data. The stability parameters of Eberhart and Russell (1966) were estimated by regressing the genotype means on to an environmental index estimated as the mean of all the genotypes in a specific environment. The regression coefficient (b_i) and deviation from regression (S^2_{di}) were the parameters used to compare the environmental responses of the genotypes. According to the Eberhart and Russell (1966) model, regression coefficients approximating unity coupled with a (S^2_{di}) value of zero indicate average stability. When this is associated with high mean yield, the genotypes have general adaptability and when associated with low mean yield, the genotypes are poorly adapted to all environments. Regression values above unity describe genotypes with higher sensitivity to environmental change and greater specificity of adaptability to high-yielding environments. Regression coefficients below unity indicate greater resistance to environmental change, and therefore more specific adaptability to low-yielding environments. The coefficient of determination (R^2) was also computed, as suggested by Pinthus (1973). A genotype with a high coefficient of determination can be considered to be stable.

The environmental variance (S^2_{xi}) of the genotypes detects all deviations from the mean (Roemer, 1917; cited in Becker and Leon, 1988). A genotype with minimum variance under different environments was considered to be stable. The superiority measure (P_i) was defined as the distance mean square between the cultivar response and the maximum response over locations (Lin and Binns, 1988). A low value of (P_i) indicates high relative stability. The mean variance component for a pair-wise GE interaction ($\overline{\Theta}_i$) was also computed, as proposed by Plaisted and Peterson (1959). This stability statistic measures a variety's contribution to the GE interaction and is computed from a total of q(q–1)/2 pair-wise analyses. In each analysis, the GE variance component is estimated. Lower values of $\overline{\Theta}_i$ indicate more stable genotypes. The GE variance component stability statistic Θ_i is the GE variance component of the experiment with variety i deleted (Plaisted, 1960). The higher the Θ_i value the more stable the genotype. Ecovalence (W^2_i), as suggested by Wricke (1962), was employed to further describe stability. The GE interaction effect for genotype i, squared and summed across all environments, is the stability measure for genotype i. A low ecovalence (W^2_i) value indicates high relative stability. An unbiased estimate using the stability variance (σ_i^2) of the genotypes was determined according to Shukla (1972). The stability was also measured by combining the coefficient of variation (CV_i) and the mean yield (Francis and Kannenberg, 1978). Genotypes with a low CV and high yield were regarded as most desirable. The yield reliability index (I_i), proposed by Kataoka (1963) for economic analysis, has the advantage of integrating yield level and yield stability into a single index of yield reliability. The relative importance attributed to yield stability in the index depends on the average level of risk aversion of farmers in the target region or sub-region. AMMI stability value (ASV) is the distance from the coordinate point to the origin in a two-dimensional scattergram of IPCA1 scores against IPCA2 scores in the AMMI model (Purchase et al., 2000). The larger the IPCA scores, either negative or positive, the more specifically adapted a genotype is to certain environments, smaller IPCA scores indicating a more stable genotype across environments. Finally, a new approach known as the Yield and Stability Index (YSI) is recommended, calculated by ranking the sum of stability indices and mean yield. Low values of this parameter show desirable genotypes with high mean yield and stability.

Results and discussion

AMMI analysis

The results of AMMI analysis for the seed yield of 16 genotypes are presented in Table 2. The differences between genotypes, environments and GE interaction were highly significant. This revealed that the agro-climatic conditions of the environments were different, and that there was a differential

response of the genotypes to environments. The percentage of the sum of squares (SS) attributable to the environment, genotype, and GE after removing SS due to error and replication was 87.1% (Table 2). The environment accounted for a high percentage of the remaining SS (76.5%). GE effects accounted for a relatively small proportion of the residual SS in comparison with the environment, but it was approximately three times larger than the genotype effect, indicating the importance of the GE interaction. AMMI analysis of variance partitioned the GE interaction into five interaction principal component axes (IPCA), all of which were significant for seed yield, while two first principle components explained 51.5% of the GE interaction. Table 3 shows the AMMI genotype selections per environment. G16 was clearly the superior genotype in 15 out of 18 environments, followed by G1, G9 and G2, which were selected in 12, 11 and 8 environments, respectively, as the first four selections.

To further investigate the interaction across environments effect, a biplot was constructed with IPCA1 and IPCA2 on the X- and Y-axis, respectively, which can be interpreted by comparing the interaction scores for each genotype and environment. Genotypes or environments with dissimilar interaction scores indicate dissimilar interaction effects, while genotypes or environments with interaction scores close to zero indicate negligible interaction effects. This graph was manually divided into three groups, showing that genotypes G7, G10, G1, G5 and G15 and environments M, C, R and N had the greatest share in the interaction, whereas genotypes G16, G13, G3, G4, G12, G11 and G2, along with environments K, L, B and Q, indicated a moderate interaction, and the rest of the genotypes and environments, which had IPCA scores near to zero, showed the least interaction effects (Fig. 1).

Table 2
AMMI analysis of seed yield for 16 safflower genotypes across 18 environments

Source	df	SS	MS	% SS explained
Total	863	1.63E+08	189348	
Treatments	287	1.42E+08	495718**	87.1
Genotypes	15	8752241	583483**	6.2
Environments	17	1.09E+08	6404209**	76.5
Interactions	255	24647256	96656**	17.3
IPCA1	31	8138300	262526**	33.0
IPCA2	29	4548986	156862**	18.5
IPCA3	27	3230016	119630**	13.1
IPCA4	25	2585030	103401**	10.5
IPCA5	23	1739171	75616**	7.1
Residuals	120	4405754	36715^{ns}	
Block	36	2250522	62515**	1.3
Error	540	18885641	34973	11.6

NB: The block source of variation refers to blocks within environments; ** Significant at the 0.01 level of probability; ns: non-significant

Table 3
Mean yield (kg ha^{-1}), IPCA scores and first four AMMI selections for each environment

Environment	Mean yield	IPC1	IPC2	First four AMMI selections			
A	975	7.10	-3.91	G16	G1	G14	G9
B	424	–8.00	–3.43	G16	G7	G9	G4
C	613	–21.92	7.98	G10	G4	G5	G3
D	847	–4.48	–0.79	G16	G9	G2	G7
E	663	–6.46	–2.22	G16	G9	G7	G4
F	240	–2.91	–0.67	G16	G9	G1	G2
G	608	–1.94	2.21	G16	G9	G1	G2
H	785	–0.83	5.81	G16	G10	G1	G2
I	602	–1.47	–5.64	G16	G1	G7	G9
J	300	–0.65	–0.26	G16	G1	G9	G2
K	472	9.97	–5.22	G16	G1	G14	G15
L	1431	11.05	6.31	G16	G1	G13	G2
M	1396	25.46	8.91	G1	G16	G13	G11
N	709	7.43	–18.16	G16	G1	G15	G12
O	511	–3.74	–4.13	G16	G9	G7	G1
P	574	–2.56	–0.53	G16	G9	G1	G2
Q	639	–5.34	–8.55	G16	G7	G9	G12
R	1466	–0.72	22.30	G10	G16	G13	G2

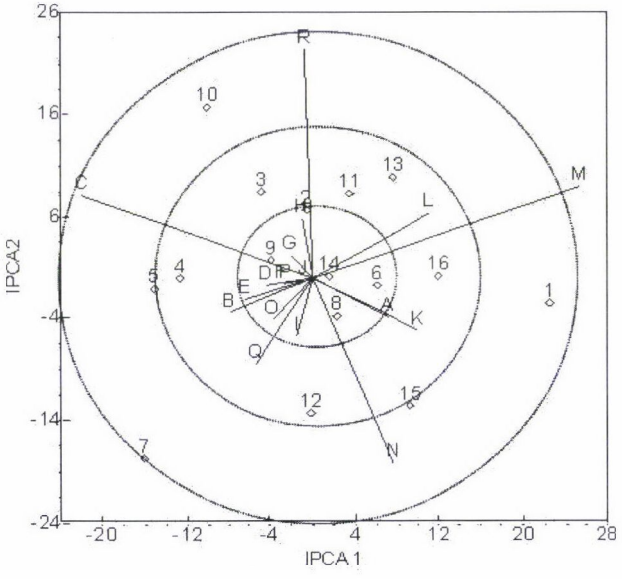

Fig. 1. Biplot of IPCA1 and IPCA2 scores for environments and genotypes

Environments D, J and P, representing the Gonbad site in all three years, were all placed in the group of environments with very low interaction effects, so it can be concluded that a one-year trial is sufficient in safflower breeding programmes in this location. The fact that the first group of environments included Sararood in 2004–05 (M), Khoram-abad in 2004–05 (R), Ghamloo in

2002–03 (C) and Ardebil in 2004–05 (N), while Shirvan in 2003–04 (K) and 2004–05 (Q) was placed in the middle group and Gonbad in 2002–03 (D), 2003–04 (J) and 2004–05 (P) in the last group indicated completely dissimilar IPCA components, i.e. these six locations are different environments representing mega-environments for seed yield in safflower breeding programmes in Iran, none of which can be neglected, though Gonbad was a stable location over the years. Genotypes G14, G8, G9 and G6 were the most stable genotypes, but on the basis of mean yield (Table 4) G9 can be selected from these genotypes as a stable genotype with relatively high mean yield.

Table 4

Mean yield and values and ranking (numbers in parenthesis) of stability parameters for 16 genotypes over environments

Genotype code	Mean yield (Y)	ASV	I_i	P_i	CV_i	S^2_{xi}	W^2_i	σ^2_i
G1	868.9(2)	47(9)	665(1)	59142(15)	60(10)	270132(16)	1048309(16)	68173(16)
G2	791.1(4)	46(8)	512(10)	73020(13)	50(5)	160252(7)	242787(1)	14020(1)
G3	744.2(7)	80(11)	507(11)	85969(10)	53(7)	156818(6)	246338(2)	14259(2)
G4	710.8(10)	23(6)	408(14)	113873(5)	44(2)	101360(3)	417426(7)	25761(7)
G5	624.5(15)	29(7)	402(15)	155502(2)	49(4)	98642(2)	518816(8)	32577(8)
G6	547.3(16)	11(3)	563(8)	183769(1)	76(14)	193724(9)	353148(6)	21440(6)
G7	689.1(11)	343(16)	348(16)	136328(4)	39(1)	73942(1)	909044(15)	58811(15)
G8	682.5(13)	19(4)	467(12)	109594(7)	52(6)	133235(5)	318352(5)	19100(5)
G9	814.3(3)	10(2)	585(6)	64697(14)	54(8)	208617(11)	694704(13)	44401(13)
G10	737.4(8)	295(15)	593(5)	102593(8)	61(11)	214604(12)	703055(14)	44963(14)
G11	723(9)	75(10)	601(3)	91581(9)	62(12)	220482(14)	295878(4)	17590(4)
G12	689.1(12)	175(13)	417(13)	110578(6)	46(3)	106251(4)	281494(3)	16623(3)
G13	752.8(6)	109(12)	624(2)	84412(11)	63(13)	237974(15)	521586(9)	32763(9)
G14	778.5(5)	3(1)	575(7)	78125(12)	56(9)	201947(10)	546195(10)	34418(10)
G15	634.3(14)	176(14)	524(9)	146391(3)	62(12)	167796(8)	547246(11)	34488(11)
G16	994.1(1)	21(5)	595(4)	16213(16)	46(3)	216240(13)	571410(12)	36113(12)

Genotype code	Mean yield (Y)	Θ_i	$\overline{\Theta}_i$	b_i	S^2_{di}	R^2	$\overline{SI}$	YSI
G1	868.9(2)	29822(16)	48998(16)	1.28(16)	54756(15)	0.81(9)	9.8	11.8(4)
G2	791.1(4)	33432(1)	23726(1)	1.02(7)	15737(3)	0.91(2)	5.0	9.0(2)
G3	744.2(7)	33416(2)	23838(2)	1.03(8)	16226(4)	0.91(2)	6.5	13.5(6)
G4	710.8(10)	32649(7)	29205(7)	0.77(3)	18544(5)	0.82(8)	7.1	17.1(9)
G5	624.5(15)	32195(8)	32386(8)	0.74(2)	22427(7)	0.77(12)	8.8	23.8(14)
G6	547.3(16)	32937(6)	27189(6)	1.08(10)	21105(6)	0.89(3)	8.3	24.3(15)
G7	689.1(11)	30446(15)	44628(15)	0.57(1)	29900(9)	0.60(13)	10.5	22.5(13)
G8	682.5(13)	33093(5)	26097(5)	0.90(5)	57725(16)	0.86(5)	7.9	20.9(12)
G9	814.3(3)	31407(13)	37904(13)	1.07(9)	42859(14)	0.79(11)	8.3	11.3(3)
G10	737.4(8)	31369(14)	38166(14)	1.10(12)	42461(13)	0.80(10)	11.1	19.1(11)
G11	723(9)	33194(4)	25392(4)	1.20(14)	12894(2)	0.94(1)	6.8	15.8(8)
G12	689.1(12)	33259(3)	24941(3)	0.81(4)	12567(1)	0.88(4)	6.5	17.5(10)
G13	752.8(6)	32182(9)	32473(9)	1.23(15)	25285(8)	0.89(3)	8.5	14.5(7)
G14	778.5(5)	32072(10)	33245(10)	1.09(11)	32841(11)	0.84(7)	7.6	12.6(5)
G15	634.3(14)	32067(11)	33278(11)	0.96(6)	34080(12)	0.80(10)	11.0	25.0(16)
G16	994.1(1)	31959(12)	34036(12)	1.16(13)	32163(10)	0.85(6)	6.8	7.8(1)

Y: genotypes mean yield (kg ha^{-1}) over environments, ASV: Purchase et al., 2000, I_i: Kataoka, 1963, P_i: Lin and Binns, 1988, CV_i: Francis and Kannenberg, 1978 , S^2_{xi}: Environmental variance, W^2_i: Wricke, 1962, σ^2_i: Shukla, 1972, Θ_i: Plaisted, 1960, $\overline{\Theta}_i$: Plaisted and Peterson, 1959, b_i: Finlay and Wilkinson, 1963, S^2_{di}: Eberhart and Russell, 1966, R^2: Pinthus, 1973, $\overline{SI}$: Average ranking of stability parameters, YSI: Yield and Stability Index.

Stability analysis

The assessment of the genotypes based on different stability measurements and genotypic mean yield is presented in Table 4. According to the method of Eberhart and Russell (1966) genotypes G2 and G3 had b_i values near to unity and low S^2_{di}, indicating an average stability over environments.

In terms of the Finlay and Wilkinson (1963) parameter, genotypes G7, G5, G4 and G12 were stable, with low values of b_i. Genotypes G1, G13, G11 and G16 had large b_i values, indicating low stability in all environments. The value of b_i showed a significant negative correlation with mean yield ($P<0.05$), P_i and I_i ($P<0.01$), and was correlated with CV_i and S^2_{xi} ($P<0.01$) (Table 5).

Following the approach of Pinthus (1973), genotypes G11, G2 and G3, which had higher coefficients of determination, were considered to be stable, while genotypes G7, G5 and G9, with lower R^2, were considered to be unstable. R^2 was significantly correlated with $\overline{\Theta}_i$ (r=0.78**), Θ_i (r=0.78**), W^2_i (r=0.78**), σ_i^2 (r=0.78**) and S^2_{di} (r=0.57*).

Based on environmental variance (S^2_{xi}), genotypes G7, G5 and G4, with minimum variance in different environments, were considered to be stable, while genotypes G1, G13 and G11 were considered as unstable. S^2_{xi} showed a significant correlation with $\overline{\Theta}_i$, Θ_i, W^2_i and σ_i^2 (r=0.72**). Using the parameter P_i (Lin and Binns, 1988) genotypes G16, G1 and G9 had low values, indicating high relative stability, and these genotypes also had high seed yield. With this same method, genotypes G6, G5 and G15 had high P_i values, showing low relative stability. P_i was significantly correlated with mean yield (r= 0.98**) and I_i (r=0.64**).

In keeping with Plaisted and Peterson (1959), genotypes G2, G3 and G12 had lower $\overline{\Theta}_i$ values, indicating high stability. The stability parameter Θ_i (Plaisted, 1960) showed results similar to the $\overline{\Theta}_i$ parameter, but with this method stable genotypes had higher Θ_i values. Based on the Wricke (1962) stability parameter, W^2_i, genotypes G1, G7 and G10 had higher values, showing them to be unstable, while genotypes G2, G3 and G12 were considered to be stable, with low values of W^2_i. In terms of stability variance (σ_i^2), genotypes G2, G3 and G12 had the smallest variance across environments, so they were stable. The results obtained for the four parameters $\overline{\Theta}_i$, Θ_i, W^2_i and σ_i^2 were the same and were significantly correlated with each other (r=1.0**), so it is sufficient to use one of them. Similar results were reported in lentil (Mohebodini et al., 2006). These parameters showed no correlation with the other parameters, making them an independent group for the assessment of stability. According to the stability measure (CV_i) of Francis and Kannenberg (1978), genotypes G7, G4, G16 and G12 were considered to be stable genotypes. Based on its low CV_i value and high mean yield, G16 is the most desirable genotype. CV_i showed a negative correlation with I_i (r=−0.69**) and a positive correlation with S^2_{xi} (r=0.69**) and b_i (r=0.68**). When applying the yield reliability index (I_i) proposed by Kataoka

(1963), which has the advantage of integrating yield level and yield stability into a single index of yield reliability, genotypes G1, G13, G11 and G16 showed the highest I_i values and can be considered as desirable genotypes. Considering the mean yield across environments, genotypes G16, G1 and G9 had the highest mean yield and G6, G5 and G15 the lowest. In terms of the AMMI stability value (ASV; Purchase et al., 2000) genotypes G14, G9 and G6 were stable. ASV exhibited no significant relationship with the other parameters.

In view of the above results, it is obvious that no genotypes having both high yield performance and high relative stability can be selected. On the other hand, the results obtained with the individual parameters differed; for example, the rank of genotype G2 according to ASV, I_i, P_i, CV_i, S^2_{xi}, W^2_i, S^2_{xi}, b_i, and R_2 ranged from 1 to 13 among the 16 genotypes evaluated, so there was great fluctuation between the stability rankings recorded using different parameters, making selection based on these parameters difficult. In addition, when calculating these parameters, attention is mainly paid to the stability aspect, but not to high yield performance. To facilitate genotype selection, an approach is thus suggested that pays attention to both mean yield and relative stability. The average stability parameter ranking ($\overline{SI}$) is considered as relative stability and the ranking of genotypes based on mean yield over environments (Y) as a parameter of genotype performance. The sum of these two rankings is considered as a final parameter, the Yield and Stability Index (YSI) where genotypes with low values are the best. Based on the YSI ranking, genotypes G16, G2, G9 and G1 were the best. These genotypes were the same as those selected using the AMMI model (G16, G1, G9 and G2), so this new approach appears to give reliable results.

Table 5
Correlation of stability parameter ranking with mean seed yield of genotypes (n=16)

	Mean yield	ASV	I_i	P_i	CV_i	S^2_{xi}	W^2_i	σ^2_i	$\overline{\Theta_i}$	Θ_i	b_i	S^2_{di}
ASV	0.19											
I_i	0.61*	0.10										
P_i	0.98**	0.25	0.64**									
CV_i	0.03	–0.01	–0.69**	–0.01								
S^2_{xi}	–0.61*	–0.10	–1.00	–0.64**	0.69**							
W^2_i	–0.25	0.12	–0.32	–0.18	0.08	0.32						
σ^2_i	–0.25	0.12	–0.32	–0.18	0.08	0.32	1.0**					
Θ_i	–0.25	0.12	–0.32	–0.18	0.08	0.32	1.0**	1.0**				
$\overline{\Theta_i}$	–0.25	0.12	–0.32	–0.18	0.08	0.32	1.0**	1.0**	1.0**			
b_i	–0.61*	–0.12	–0.98**	–0.64**	0.68**	0.98**	0.24	0.24	0.24	0.24		
S^2_{di}	–0.19	–0.20	–0.29	–0.24	0.20	0.29	0.72**	0.72**	0.72**	0.72**	0.21	
R^2	0.12	0.12	0.26	0.20	–0.28	–0.26	0.78**	0.78**	0.78**	0.78**	–0.36	0.57*

ASV: Purchase et al., 2000, I_i: Kataoka, 1963, P_i: Lin and Binns, 1988, CV_i: Francis and Kannenberg, 1978, S^2_{xi}: Environmental variance, W^2_i: Wricke, 1962, σ^2_i: Shukla 1972, Θ_i: Plaisted, 1960, $\overline{\Theta_i}$: Plaisted and Peterson, 1959, b_i: Finlay and Wilkinson, 1963, S^2_{di}: Eberhart and Russell, 1966, R^2: Pinthus, 1973

Conclusions

Multi-environment trials (MET) are the most acceptable method for genotype recommendation and the determination of mega-environments in crop production regions because of the existence of GE interactions. There are many methods, indices and parameters for the analysis of MET data for these purposes. Genotype recommendation is divided into two aspects, namely high yield performance and yield stability. Many parameters and indices have been introduced to determine stable genotypes, but the results obtained with these parameters are different. On the other hand, yield stability is usually correlated with low mean yield, so the introduction of methods considering both aspects could be useful for genotype recommendation. All the mentioned goals can be achieved using AMMI analysis, as demonstrated in the present study (using Genstat and IRRISTAT software). The Yield and Stability Index (YSI) approach is easy to apply and calculate, based on stability indices and genotype mean yield, and could be of service in genotype recommendation.

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PLANT DIVERSITY AND SPECIES RICHNESS OF LJUBLJANA MARSH GRASSLANDS UNDER THE INFLUENCE OF DIFFERENT CUTTING AND FERTILIZING REGIMES

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The botanical composition of grasslands determines the agronomic and natural values of swards. Good grassland management usually improves herbage value, but on the other hand it frequently decreases the plant diversity and species richness in the swards. In 1999 a field trial in a split-plot design with four replicates was therefore established on the *Arrhenatherion* type of vegetation in Ljubljana marsh meadows in order to investigate this relationship. Cutting regimes (2 cuts – with normal and delayed first cut, 3 cuts and 4 cuts per year) were allocated to the main plots and fertiliser treatments (zero fertiliser – control, PK and NPK with 2 or 3 N rates) were allocated to the sub-plots. The results at the 1st cutting in the 5th trial year were as follows: Fertilising either with PK or NPK had no significant negative effect on plant diversity in any of the cutting regimes. In most treatments the plant number even increased slightly compared to the control. On average, 20 species were listed on both unfertilised and fertilised swards. At this low to moderate level of exploitation intensity, the increased number of cuts had no significant negative effect on plant diversity either (19 species at 2 cuts vs. 20 species at 3 or 4 cuts). PK fertilisation increased the proportion of legumes in the herbage in the case of 2 or 3 cuts. The proportion of grasses in the herbage increased in all the fertilisation treatments with an increased numbers of cuts. Fertiliser treatment considerably reduced the proportion of marsh horsetail (*Equisetum palustre*) in the herbage of the meadows. This effect was even more pronounced at higher cut numbers. The proportion of *Equisetum palustre* in the herbage was the highest in the unfertilised sward with 2 cuts (26.4 %) and the lowest in the NPK-fertilised sward with 4 cuts (1.4%).

Key words: grassland, botanical composition, cutting, fertilising, Shannon diversity index, species richness, marsh horsetail, Ljubljana marsh

Introduction

Over the last fifty years approximately 2700 km^2 of abandoned Slovene grasslands have been taken over by forest vegetation. On the managed grasslands in Slovenia the three-cut and two-cut systems (karst and wet grasslands) dominate.

The Ljubljana marsh can be described as environmentally sensitive. Considerable attempts were made in the past to turn this marsh into a landscape park, but so far without success.

The phytocenological associations on the Ljubljana marsh area suitable for grassland farming are: *Arrhenatherion*, *Molinion* and *Filipendulion*. Approximately 75% of the Ljubljana marsh area (160 km^2) is covered by semi-natural grasslands of the *Arrhenatherion* type. One of the major problems with fodder production on this type of grasslands is the massive appearance of marsh horsetail (*Equisetum palustre*). The second type of grasslands, with a much smaller area, is the *Molinion* type of vegetation.

Managing grasslands on Ljubljana marsh has a relatively long tradition. Many young farmers on Ljubljana marsh have abandoned farm activities to look for employment in the nearby city of Ljubljana. Due to the fact that the Ljubljana marsh area is environmentally sensitive, the main reason for the present work was to establish management strategies for grassland fodder production on Ljubljana marsh with minimal negative effects on the environment (Čop et al., 2004a; b). Relatively moderate fertilizer treatments were used and non-intensive cutting regimes were chosen in the trials. The present study investigated the effects of cutting and fertilizer treatments on the botanical composition (Sanderson et al., 2005; 2007) of some Ljubljana marsh swards. The effects of the treatments on the plant diversity and species richness of the grass sward were also studied.

Materials and methods

A field trial was established in March 1999 on semi-natural *Arrhenatherum elatius* grassland on Ljubljana marsh (lat. 45° 58′ N, long. 14° 28′ E, alt. 295 m) in a split-plot design with four replications, with cutting regimes in the main plots and the four fertilizer treatments in the subplots. The size of the sub-plots was 2.5 × 4 m. The cutting regimes were: 2 cuts with a delayed first cut, 3 cuts and 4 cuts per year. The fertilizer treatments were 0 (Zero), PK (35 kg P + 133 kg K ha^{-1} yr^{-1}); N_1 PK (50 kg N ha^{-1} applied to first cut only + 35 kg P and 133 kg K ha^{-1} yr^{-1}); N_cPK (50 kg N ha^{-1} applied to each of 2, 3 or 4 cuts + 35 kg P and 133 kg K ha^{-1} yr^{-1}).

The soil on the plot was pH neutral (7.2), with low P and K content (ammonium lactate extraction; P = 0.9–2.2 mg, K = 7.7– 9.0 mg per 100 g dry soil).

The results presented are from the first cut of the fifth trial year (May 11 to June 25, 2003) and consist of the ratio of various botanical groups (and of *Equisetum palustre*) in the herbage (Table 1), Shannon plant diversity index, plant number and species richness. (Table 2). Plant species diversity usually refers to the number of species (richness) and their relative abundance (evenness) within a defined area (Soder et al., 2007).

The botanical survey of the *Arrhenatherum elatius* grassland on Ljubljana marsh was carried out with the Braun-Blanquet (1964) method, for each cutting regime and fertilizer treatment. The plants were determined using the nomenclature of Martinčič et al. (1999) and Seliškar (1986). The Braun-Blanquet method involves identifying all plant species represented on the research area (plot), then assigning each a code based on its contribution to the total area.

Table 1

Proportion of botanical groups and *Equisetum palustre* (% of herbage fresh matter) in *Arrhenatherum elatius* grassland in the first cut of the 5th trial year for each cutting regime and fertilizer treatment

Fertilizer	Cutting regime	Grasses	Legumes	Herbs	*E. palustre*
Zero	2 cuts (delayed)	55.8	0.5	43.7	26.4
Zero	3 cuts	75.7	0.6	23.7	14.8
Zero	4 cuts	73.0	0.3	26.7	10.3
PK	2 cuts (delayed)	75.5	1.9	22.6	7.6
PK	3 cuts	83.3	3.3	13.4	4.0
PK	4 cuts	82.3	0.5	17.2	2.5
N_1PK	2 cuts (delayed)	85.2	1.9	12.9	6.6
N_1PK	3 cuts	86.9	0.4	12.7	3.8
N_1PK	4 cuts	87.5	0.6	11.9	1.4
N_cPK	2 cuts (delayed)	82.3	0.7	17.0	7.5
N_cPK	3 cuts	86.3	0.7	13.0	3.1
N_cPK	4 cuts	92.9	0.0	7.1	1.6

Fertilizer treatments were 0 (Zero), PK (35 kg P + 133 kg K ha^{-1} yr^{-1}); $N_{1)}$PK (50 kg N ha^{-1} applied to first cut only + 35 kg P and 133 kg K ha^{-1} yr^{-1}); N_cPK (50 kg N ha^{-1} cut^{-1} applied to each of 2, 3 or cuts + 35 kg P and 133 kg K $ha^{-1}yr^{-1}$).

The first part of the code is based on the abundance of the plant species:

+ Very rare plant species with insignificant cover.
1. Plant species with less than 10% cover.
2. Plant species with 10–25% cover.
3. Plant species with 25–50% cover.
4. Plant species with 50–75% cover.
5. Plant species with 75–100% cover.

The second part of the code is based on the sociability of the plant species:

1. Plant species growing alone.
2. Plant species growing in tufts.
3. Plant species forming pads
4. Plant species forming groups
5. Plant species forming carpets.

Results

The presented results are from the first cut of the fifth trial year (May 11 to June 25, 2003) from a trial on Ljubljana marsh area, based on the *Arrhenatherion* type of vegetation, and consist of the proportion of botanical groups (and *Equisetum palustre*) in the herbage, based on Shannon plant diversity index, plant number and species richness.

The grassland community in the trial consisted of approximately 30 plant species, with *E. palustre* and *A. elatius* prevailing. After fertilizer use in the first year, there was a change in species composition. The ratio of botanical groups (grasses, legumes, herbs and *Equisetum palustre*), measured in the fifth trial year (first cut), showed that the grass sward was less affected by cutting ($P < 0.003$) than by fertilizer application ($P < 0.001$). Compared to the control plots, the

proportion of grasses in all the fertilized swards increased. This was most evident in the regime with four cuts (Table 1).

Plant species diversity (Sanderson et al., 2004) and species richness (Tracy and Faulkner, 2006) were also studied in the trial. Intensification did not negatively affect sward plant diversity (Table 2), which was stable and relatively high, with the highest values in the 4-cut regime. These results contradict findings in the literature indicating a negative relationship between fertilizer treatments and plant diversity (Ellenberg, 1952; Nösberger et al., 1994, Zechmeister et al., 2003) (Table 2).

Shannon plant diversity index, plant number and species richness were recorded. Increased plant species diversity has been linked to improvements in ecosystem functions such as nutrient retention and resistance to weed invasions. The plants may have adapted to human activity, as there is a relatively long tradition of farming on Ljubljana marsh. Over many years the genotypes of the plants may be affected and adapted to the relatively long period of management, especially to the cutting regime. On the other hand, unmanaged grasslands and meadows on Ljubljana marsh are important for bird survival during migration and nesting.

The results of the botanical survey on the *Arrhenatherion* grassland for the different fertilizer and cutting regimes are presented in Table 3.

Discussion

In a five-year trial on Ljubljana marsh, increasing the frequency of cutting and the use of inorganic fertilizer improved the agronomic value of the managed *Arrhenatherion* grass sward. It also maintained plant diversity in the grassland at the level of the less intensively managed sward.

Table 2

Shannon diversity index and species richness (species number) in the *Arrhenatherum elatius* grassland in the first cut of the 5th trial year for each cutting regime and fertilizer application

Cutting regime	Shannon diversity index†					Species richness‡				
	Zero	PK	N_1PK	N_cPK	Mean	Zero	PK	N_1PK	N_cPK	Mean
2 cuts (delayed)	1.92	1.42	1.43	1.52	1.57a§	19	15	14	15	16a
3 cuts	2.11	2.06	1.94	1.80	1.98b	22	26	21	20	22b
4 cuts	2.35	2.16	2.18	1.70	2.10b	23	21	23	20	22b
Average	2.13b§	1.88a	1.85a	1.67a	1.88	21a	21a	19a	18a	20

† $P = 0.006$ for cutting; $P = 0.003$ for fertilizing; no cutting × fertilizing interaction; ‡ $P = 0.001$ for cutting; P = 0.138 for fertilizing; no cutting × fertilizing interaction; § Means within a column or row followed by the same letter are not significantly different at $P = 0.05$ according to Duncan's multiple range test.

Table 3
Botanical survey of the *Arrhenatherum elatius* grassland (Braun-Blanquet method) for each cutting regime and fertilizer application (5th trial year)*

Plant species	2 cuts – delayed				3 cuts				4 cuts			
	Zero	PK	N_1PK	N_cPK	Zero	PK	N_1PK	N_cPK	Zero	PK	N_1PK	N_cPK
Anthoxanthum odoratum								1.1	+.1	+.1	+.1	+.1
Arrhenatherum elatius	2.1	2.1	2.1	3.1	+.1	3.1	3.1	3.1	+.1	2.1	1.1	3.1
Dactylis glomerata	1.1	+.1	+.1	1.1	+.1	1.1	1.1	1.1	+.1	1.1	1.1	1.1
Festuca pratensis	+.1	+.1			1.1	1.1	1.1	1.1		1.1	1.1	+.1
Festuca rubra agg.	2.1	1.1	1.1	+.1	2.1	1.1	1.1		3.1	1.1	3.1	+.1
Helictotrichon pubescens			+.1	+.1	2.1	1.1	2.1	2.1	1.1	1.1	1.1	1.1
Holcus lanatus	+.1	1.1	1.1	+.1							+.1	
Poa trivialis	+.1						+.1	+.1				1.1
Lathyrus pratensis	+.1	1.1	+.1	+.1		+.1		+.1				
Medicago lupulina		+.1								+.1		+.1
Trifolium pratense		+.1			1.2	1.2	1.2					+.1
Vicia cracca	+.1	+.1		+.1	+.1	+.1	+.1	+.1		+.1	+.1	+.1
Achillea millefolium agg.	+.1	+.1	+.1	+.1	1.1	+.1	2.1	1.1	+.1	+.1	+.1	1.2
Ajuga reptans					+.1				+.1			+.1
Angelica sylvestris		+.1				+.1	+.1			+.1		
Calystegia sepium		+.1	+.1	+.1			+.1					
Campanula patula	+.1		+.1	+.1		+.1	+.1			+.1		
Centaurea jacea	+.1	+.1	+.1	+.1	+.1	+.1	+.1	+.1	+.1	+.1	1.1	+.1
Cerastium holosteoides								+.1			+.1	+.1
Cirsium oleraceum		+.1	+.1				+.1				+.1	
Convolvulus arvensis	+.1	+.1	+.1	+.1		+.1	+.1			+.1		+.1
Cruciata glabra		+.1	+.1	+.1	.	+.1				+.1		
Daucus carota	+.1		+.1	+.1	+.1			+.1				
Equisetum palustre	3.1	1.1	1.1	+.1	3.1	1.1	+.1	+.1	3.1	+.1	+.1	+.1
Erigeron annuus							+.1	+.1		+.1		
Galium mollugo	1.1	2.1	2.1	2.1	1.2	2.1	1.1	2.1	1.1	+.1	+.1	1.1
Glechoma hederacea				+.1		+.1						0.1
Leontodon hispidus	+.1				+.1	+.1			+.1	+.1		
Leucanthemum ircutianum	+.1	+.1	+.1	+.1	+.1	+.1	+.1	+.1	+.1	1.1	2.1	+.1
Lythrum salicaria	+.1	+.1		+.1	+.1		+.1					
Silene latifolia		+.1		+.1	+.1	+.1	+.1	+.1				
Mentha aquatica	+.1	+.1				+.1			+.2			+.2
Mentha longifolia			+.1	+.1				+.1				
Pastinaca sativa	+.1	+.1	+.1	+.1	+.1		+.1	+.1				
Pimpinella major	+.1	+.1	+.1							+.1	+.1	
Plantago lanceolata	+.1				1.1	+.1	1.1	1.1	1.1	+.1	+.1	+.1
Ranunculus acris	+.1	+.1			1.1		1.1	1.1	+.1	+.1	+.1	+.1
Ranunculus repens	+.1	+.1	+.1	+.1	+.1	+.1	+.1	+.1	+.1	+.1	+.1	+.1
Rumex acetosa							+.1	+.1	+.1		+.1	
Taraxacum officinale								+.1	+.1	+.1		+.1
Verbascum sp.				+.1		+.1	+.1		+.1			
Veronica persica	+.1			+.1			+.1		+.1	+.1	+.1	+.1
Total number of species	28	28	23	28	25	28	29	29	28	26	24	27

*Species that cover less than 1%, appearing in one or two treatments only, are not included in the table; For explanation of codes, see Materials and methods

The management system adopted in the trial also drastically decreased the occurrence of the poisonous marsh horsetail (*Equisetum palustre*), which is wide-spread on *Arrhenatherum* grasslands on Ljubljana marsh and represents a major problem for grassland farming in this area. In the trial PK fertilizers increased the proportion of legumes in the herbage after 2 or 3 cuts. The proportion of grasses in the herbage increased in all the fertilizer treatments with an increased number of cuts. The grass sward was less affected by cutting than by fertilizer application.

The intensification adopted in the trial (fertilizing and cutting) had no negative effect on plant species richness or plant diversity, compared to the control plots. Instead of stress-tolerant plant species, the management system resulted in a larger number of competitive plant species. These results contradict data found in the literature, indicating a negative correlation between grassland management and the plant diversity and plant richness of swards. This is probably due to the moderate exploitation level in the trial. Relatively moderate fertilizer treatments were used and less intensive cutting regimes were applied.

Grassland farmers face many challenges in management, including improving sustainability, reducing inputs of fertilizers and pesticides and protecting soil resources. Good grassland management usually improves herbage value, but on the other hand frequently decreases the plant diversity and plant species richness of the swards.

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PHYSICOCHEMICAL PROPERTIES OF THE SOIL, AND THE TOXICITY OF HEAVY METALS TO RHIZOBIA INFECTING PEA AND EGYPTIAN CLOVER IN SOIL AND LIQUID CULTURE

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Soil samples were collected from 13 locations in Haryana, after irrigation with sewage water for the past 10–15 years, and were analysed for heavy metals (Cd, Cu, Zn and Ni) and physicochemical properties, such as pH, EC, C, N, and total and available (DTPA-extractable) P. The total heavy metal contents in the soils ranged from 1.3–6.7 mg kg^{-1} for Cd, 55.8–353.2 mg kg^{-1} for Cu, 356–1028 mg kg^{-1} for Zn and 90.0–199.7 mg kg^{-1} for Ni. Though soil sample size was small, a significant negative correlation was observed between the organic C and Ni contents in the soil (r^2= –0.870, P=0.01). The survival of *Rhizobium leguminosarum* bv. *viciae* (strain PRH 1) and *R. leguminosarum* bv. *trifolii* (B 48) tagged with green fluorescent protein (*gfp*) was monitored in liquid culture as well as in the above soil samples. In liquid culture the order of heavy metal toxicity for both strains was Cd>Ni>Cu≥Zn. Soils receiving sewage water showed a 25–97% reduction in the viable cell number of *Rhizobium leguminosarum* strains. Available Cd showed a positive correlation and the other three metals a negative correlation with the reduction in cell numbers in both the strains.

Key words: heavy metals, sewage water, nodulation, nitrogen fixation, *Rhizobium leguminosarum*, survival, toxicity

Introduction

The age-old practice of using sewage water for irrigation not only enriches the soil with lost nutrients, but also allows city waste to be disposed of in an economical way. However, sewage water or sludge, particularly from industrial areas, often contains considerable amounts of potentially toxic metals such as Cu, Zn, Ni, Cd, Pb and Cr (Giller et al., 1998). Therefore, continuous irrigation with sewage may build up these toxic elements in the soil to a level that is injurious for the health of not only soil and plants but also soil microflora and fauna. As a consequence, several microbial processes, including nitrogen fixation and the degradation of xenobiotics in soil, are adversely affected

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(Ibekwe et al., 1995; Smith and Giller, 1992; Smith, 1997; Chaudri et al., 2000; Dudeja et al., 2002; Chaudhary et al., 2004; Broos et al., 2004; 2005). Keeping this in view, studies were made on the physicochemical properties of various soils which had received sewage irrigation for the last 10–15 years to determine the levels of toxic heavy metals. The survival of *Rhizobium leguminosarum* bv. *trifolii* and bv. *viciae* at these heavy metal levels was examined both *in situ* and *in vitro*.

Materials and methods

Soil samples

Soil samples were collected from farmer's fields at 13 locations in Haryana, which have been receiving irrigation from sewage water for the past 10–15 years. A composite soil sample from 6 sites up to a depth of 15 cm was collected from two locations each in Rohtak, Sonipat, Panipat, Jagadhari and Ballabhgarh and from 3 locations in Hisar. In these locations, because of industrial activities, a large quantity of toxic metals is discharged into the sewage. A soil sample collected from a research field of the CCS Haryana Agricultural University in Hisar (dry land area), to which sewage water had never been added, was used as a control soil for comparison purposes. A portion of each soil sample was air dried, powdered, passed through a 2 mm sieve and used for chemical analysis, while the other portion was used to study the survival of rhizobia.

Soil analysis for heavy metals

Total heavy metals (Cd, Cu, Zn, Ni) were extracted by digesting the finely ground soil with aqua regia (McGrath and Cunliffe, 1985) and the available metals were extracted using the DTPA method (Lindsay and Norvell, 1978). The metals were estimated using an atomic absorption spectrophotometer.

Physicochemical analysis of the soil

For the estimation of pH and electrical conductivity, each soil sample was mixed with distilled water in a ratio of 1:2 or 1:2.5 and shaken at room temperature for 2 or 24 h, respectively. Standard methods were used to analyse the soils for organic C, total N (Bremner, 1965), total P (John, 1970) and available P (Olsen et al., 1954).

Bacterial strains

Rhizobium leguminosarum bv. *viciae* PRH-1 was obtained from the Department of Microbiology, CCS Haryana Agricultural University, Hisar and *R. leguminosarum* bv. *trifolii* B-48 from the Department of Genetics.

Tagging of R. leguminosarum *strains with* gfp

Both the strains of *Rhizobium* were tagged with green fluorescent protein (*gfp*) by biparental patch-mating (Simon, 1984) as described earlier (Dudeja et al., 2002). The transconjugants giving maximum fluorescence under UV light at 395 nm were selected.

Screening of R. leguminosarum *strains for their sensitivity to heavy metals*

The sensitivity of *R. leguminosarum* strains to heavy metals was determined by inoculating 1 mL culture of each strain into yeast extract mannitol (YEM) broth containing different concentrations of heavy metals (Cd, Cu, Zn and Ni). Stock solutions of heavy metals were prepared by dissolving their respective chlorides or sulphates in sterile distilled or deionized water, followed by filter sterilization. The concentrations were selected on the basis of directives from the

European Union (EU), and were above and below the permissible limits. The growth and survival of the two marked strains PRH-1 (*gfp*) and B-48 (*gfp*) were observed for 15 d in YEM broth containing heavy metals at different concentrations. The inhibition of growth by the heavy metals was also observed under solid culture conditions. The rhizobial strains were spread on YEMA plates containing kanamycin and naladixic acid (25 and 50 μg mL^{-1}, respectively) and solutions with different concentrations of heavy metals were filled into wells made in the agar medium. The inhibition of growth was measured after 2–4 d of incubation.

Survival of gfp-*tagged* Rhizobium leguminosarum *strains in soils receiving sewage water*

To study the survival of *R. leguminosarum* strains, 100 g sieved soil from each composite field sample was placed in polyethylene bags, inoculated with equal cell populations of each *Rhizobium* strain (3.6×10^{11} cfu of strain PRH-1 and 5.13×10^{11} cfu of strain B-48) and incubated at 30°C. Viable counts were made on YEMA containing kanamycin and nalidixic acid after 0, 5, 10, 15, 30 and 45 d of incubation. Rhizobial colonies were confirmed by the presence of *gfp* fluorescence.

Results and discussion

The analyses of total and available heavy metals (Cd, Cu, Zn and Ni) in soil samples collected from 13 locations revealed that the Cd content ranged from 1.3–6.7 mg Cd kg^{-1} soil (Table 1), indicating that the Cd content was higher in some soils and lower in others than the limits prescribed by the EU. The level of available Cd ranged from 0.09–1.95 mg Cd kg^{-1} soil. The total Cu contents in the soils ranged from 55.8–353.2 mg kg^{-1} soil and the available Cu from 6.2–47% of the total Cu content. All the soils, including the control soil, showed Zn levels above the EU permissible limit of 150–300 mg kg^{-1} soil, with concentrations ranging from 356–1028 mg Zn kg^{-1} soil, of which 2.4–13.5% was available to plants. The Ni contents in the soils ranged from a maximum of 199.7 mg kg^{-1} soil to a minimum of 90 mg Ni kg^{-1} soil, 2–9% of which was available.

In all the soil samples receiving sewage water, the pH ranged from 6.51 to a maximum of 8.64 (Table 2). The EC in the soils varied from 0.35 to 4.09 dS m^{-1} and the EC of the control soil was 0.22 dS m^{-1}. An increase of 0.1–4.15% in the organic C content was observed in some soils after sewage water application. The total nitrogen contents in the soils ranged from 0.02–0.46% and the total P contents from 250–1438 mg P kg^{-1} soil, while the available P contents varied from 5.3 to 86.5 mg P kg^{-1} soil. The total heavy metal contents and the physicochemical properties showed no significant correlation (Table 3). Though the number of soil samples in the study was very low, a significant correlation was observed between available Cd and total P ($r^2 = 0.821$, $P = 0.01$) and a negative correlation between available soil Ni and organic C ($r^2 = -0.870$, $P = 0.01$).

Table 1
Total and available heavy metal contents in soils collected from farmer's fields receiving sewage water

Locations	Total heavy metals (mg kg^{-1})				Available heavy metals (mg kg^{-1})			
	Cd	Cu	Zn	Ni	Cd	Cu	Zn	Ni
Control soil	1.7	90	356	90	0.09	13.4`	20.6	6.5
Ballabhgarh-1	4.0	245	631	191	0.17	18.9	29.6	5.3
Ballabhgarh-2	4.3	56	422	167	1.14	14.4	26.9	4.1
Sonipat-1	1.3	100	563	194	0.05	45.2	61.5	12.0
Sonipat-2	4.3	186	776	200	0.10	23.2	105.0	17.8
Panipat-1	1.3	230	475	179	0.19	14.2	24.2	4.1
Panipat-2	3.0	77	392	157	0.10	5.7	20.4	4.2
Jagadhari-1	3.0	151	992	187	0.15	72.0	68.4	4.8
Jagadhari-2	5.0	197	789	185	0.05	28.7	28.6	5.2
Rohtak-1	6.7	353	1028	178	1.95	22.2	68.3	4.5
Rohtak-2	4.0	223	686	171	0.62	49.6	26.1	3.8
Hisar-1	2.7	167	518	161	0.13	10.0	12.6	3.7
Hisar-2	3.3	124	566	185	0.21	15.1	13.6	3.5
Hisar-3	1.7	57	544	152	0.17	3.3	19.7	5.2
Hisar-4	3.7	96	439	179	0.17	15.0	23.4	4.8

Table 2
Physicochemical properties of soils collected from farmer's fields receiving sewage water

Locations	pH	EC (dS m^{-1})	Organic C (%)	Total N (%)	Total P (mg kg^{-1})	Available P (mg kg^{-1})
Control soil	7.90	0.22	0.10	0.01	320	14.3
Ballabhgarh-1	7.95	1.18	2.53	0.27	468	28.7
Ballabhgarh-2	7.41	0.45	0.78	0.11	500	67.8
Sonipat-1	7.90	1.41	3.60	0.24	625	7.7
Sonipat-2	8.06	2.31	0.34	0.06	438	17.0
Panipat-1	7.91	0.72	0.42	0.03	250	5.3
Panipat-2	7.95	0.72	0.17	0.02	313	11.2
Jagadhari-1	6.51	0.58	0.58	0.05	313	86.5
Jagadhari-2	6.76	0.44	0.29	0.02	250	7.2
Rohtak-1	7.95	4.09	4.15	0.46	1438	74.5
Rohtak-2	8.64	0.78	1.40	0.17	728	9.3
Hisar-1	9.74	11.45	0.64	0.91	500	74.3
Hisar-2	8.31	2.15	0.38	0.05	938	32.8
Hisar-3	7.67	1.92	0.42	0.04	688	14.8
Hisar-4	7.90	0.35	0.37	0.05	638	19.8

Tests for metal toxicity showed that Cd inhibited the growth of *R. leguminosarum* bv. *viciae* strain PRH-1 even at 1 μg mL^{-1} concentration under liquid culture conditions (Fig. 1a). Rather than increasing, the cell population showed a slight decline over time. At 20 μg Cd mL^{-1} medium, no rhizobial population was detectable even after 2 d of incubation. *R. leguminosarum* bv. *trifolii* B-48 was found to be slightly more resistant to Cd than *R. leguminosarum* bv. *viciae* strain PRH-1, since its growth at 1 μg ml^{-1}

concentration was comparable to that of the control. However, at higher concentrations the inhibition pattern was similar to that of strain PRH-1. In the case of Cu, no inhibitory effect on strain PRH-1 was seen at lower concentrations (5 and 20 μg Cu mL^{-1} medium, Fig. 1b). However, at 50 μg Cu mL^{-1} there was only a small increase on the first 2 d of incubation, while at 150 μg Cu mL^{-1} there was a decline immediately after inoculation. The inhibitory effect of different levels of Cu on the other strain, B-48, was similar to that for strain PRH-1. Zn was found to have a profound inhibitory effect on PRH-1, with no detectable growth at 100 μg Zn mL^{-1} medium (Fig. 1c). Strain B-48 was also inhibited at higher concentrations of Zn, but was more resistant than PRH-1. While PRH-1 was found to be resistant to 1 μg Ni mL^{-1} medium, concentrations ≥ 2 μg Ni mL^{-1} became increasingly toxic to both PRH-1 and B-48, with no detectable cells at 100 μg Ni mL^{-1} (Fig. 1d).

The effect of heavy metals was also observed on solid medium by measuring the diameter of the inhibition zone (Table 4). Although Cd was found to be toxic in liquid culture, it did not show a corresponding inhibitory effect on solid medium. With increasing concentrations of the heavy metals Cu, Zn and Ni, an increase in the inhibition zone was observed.

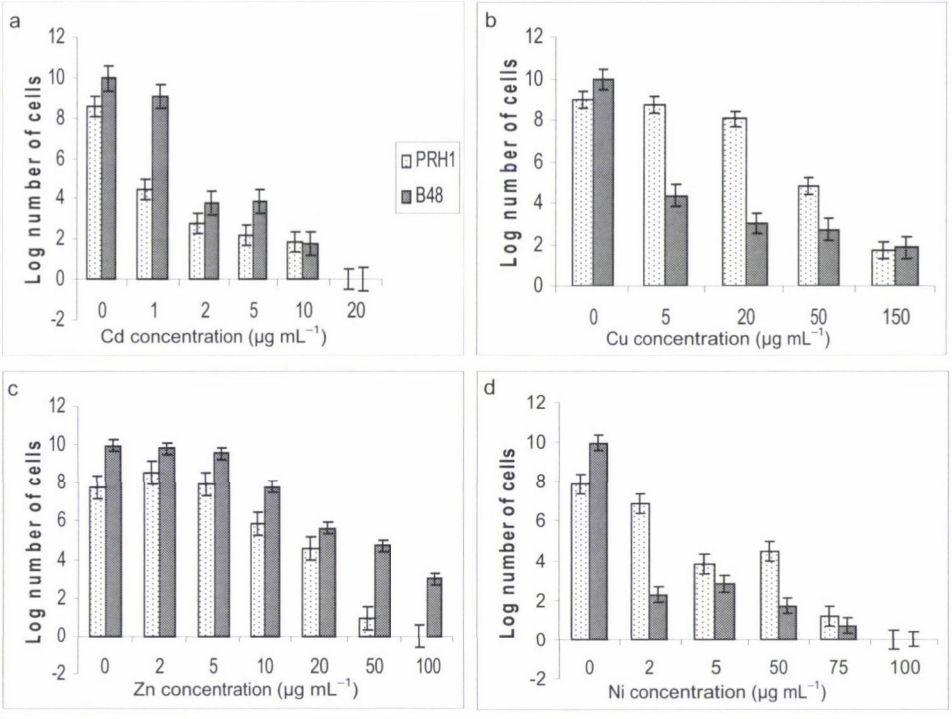

Fig 1. Effect of heavy metals Cd (a), Cu (b), Zn (c) and Ni (d) on the growth/survival of *Rhizobium leguminosarum* in liquid culture during 15 days of incubation

Table 3
Correlation coefficients between total heavy metal contents and the physicochemical properties of Haryana soils receiving sewage water

Heavy metals	pH	EC	Organic C	Total N	Total P	Available P
Total						
Cd	0.044	–0.127	0.047	0.154	0.486	0.364
Cu	0.218	0.119	0.298	0.366	0.398	0.186
Zn	0.076	–0.336	0.098	0.137	0.374	0.432
Ni	–0.001	–0.146	0.058	0.049	0.157	0.083
Available						
Cd	0.182	0.104	0.244	0.329	0.821**	0.370
Cu	–0.226	–0.383	–0.047	–0.088	–0.046	0.225
Zn	–0.065	–0.277	–0.044	–0.044	0.140	0.179
Ni	–0.066	–0.038	–0.870**	–0.128	–0.121	–0.278

** Significant at the 1% level

Table 4
Effect of heavy metals on the growth of *Rhizobium leguminosarum* bv. *viciae* PRH-1 and bv. *trifolii* B-48 on yeast extract mannitol agar containing different concentrations of heavy metals

Heavy metals	Concentrations (mg kg^{-1})	Zone of inhibition (diameter in mm)	
		R. leguminosarum bv. *viciae*	*R. leguminosarum* bv. *trifolii*
Cd	1	0	0
	2	0	0
	3	0	0
	5	0	0
	10	0	0
	20	13	11
Cu	50	14	30
	100	28	36
	150	38	46
	200	48	50
Zn	25	0	0
	50	10	0
	100	12	0
	150	12	12
	200	16	12
Ni	2	0	0
	5	0	0
	10	0	0
	25	10	0
	50	20	14
	75	22	16
	150	32	24

It is well known that some heavy metals are essential for the growth of microorganisms, but neither PRH-1 nor B-48 showed any stimulation by Cu, Cd, Zn or Ni in liquid culture. An inhibitory effect was observed as the concentration

of heavy metals increased. The stimulatory effects of traces of Zn on the growth of *Rhizobium* (Wilson and Reisenauer, 1970), and of Ni on *Bradyrhizobium japonicum* (Klucas et al., 1983) have been reported. In both strains of *R. leguminosarum,* the following order of toxicity was observed: Cd > Ni > Cu ≥ Zn, whereas other workers found an order of Cu > Cd > Zn = Ni for effective isolates and Cu > Cd > Ni > Zn for ineffective isolates of *R. leguminosarum* bv. *trifolii* (Chaudri et al., 1992). The discrepancy in the results may be due to the use of different strains.

The effect of heavy metals from sewage water irrigation on free-living rhizobia has been extensively studied and measured using various parameters such as CFU, MPN, microbial biomass, CO_2 evolution, enzyme assays, etc. After the addition of 3.6×10^{11} cells g^{-1} soil of *gpf*-tagged *R. leguminosarum* bv. *viciae* strain PRH-1 to all 13 soils, followed by incubation for 2 h, the recovery of rhizobia varied in different soils (Table 5). A reduction of 5–68% was observed in cfu for strain PRH-1 after incubation for 5 d, and a reduction of 39–97% after 45 d of incubation. *R. leguminosarum* bv. *trifolii* strain B-48 showed a 25–65% reduction in cell population in different soils, though in Jagadhari-1 and Hisar-4 soils there was only a slight reduction.

Correlation studies between the reduction in cell population in both the strains and concentrations of heavy metals showed that the percentage reduction was positively correlated in the case of available Cd ($r^2 = 0.613$ at P=0.05), while the other three available metals showed a negative correlation with the percentage reduction in the cell number of both the strains. Total P was positively correlated with a reduction in the number of rhizobia as well as with Cd contents.

Table 5

Survival of *gfp*-tagged *R. leguminosarum* bv. *viciae* and bv. *trifolii* after 45 days of incubation in Haryana soils receiving sewage water

Location	*R. leguminosarum* bv. *viciae*		*R. leguminosarum* bv. *trifolii*	
	Log No. of cells	Reduction (%)	Log No. of cells	Reduction (%)
Control soil	8.36	39	9.14	28
Ballabhgarh-1	10.67	53	10.78	52
Ballabhgarh-2	9.32	41	9.25	53
Sonipat-1	10.53	60	10.15	42
Sonipat-2	8.92	45	9.11	53
Panipat-1	8.26	39	8.69	47
Panipat-2	9.56	46	9.63	51
Jagadhari-1	7.70	28	7.85	25
Jagadhari-2	8.15	41	9.98	65
Rohtak-1	8.11	97	8.41	61
Rohtak-2	9.79	78	9.07	49
Hisar-1	8.08	100	8.51	100
Hisar-2	9.76	63	9.78	58
Hisar-3	10.04	51	10.32	53
Hisar-4	8.20	41	8.13	25

The effect of the heavy metals present in the various soils due to irrigation with sewage water reduced the number of colony-forming units of *Rhizobium leguminosarum* bv. *viciae* strain PRH-1 and *Rhizobium leguminosarum* bv. *trifolii* strain B-48. Heavy metal toxicity was observed in Rohtak-1 soil, where the number of PRH-1 (*R. leguminosarum* bv. *viciae*) decreased by 97% after 45 days of incubation, which could be due to the highest amounts of Cd, Cu and Zn in this soil. PRH-1 also showed a marked decrease in Rohtak-2 soil, which could be because of the comparatively higher availability of Cd and Cu in this soil. On the other hand, *R. leguminosarum* bv. *trifolii* strain B-48 showed better survival in Rohtak-1 and -2 soils, indicating that this strain is more tolerant of heavy metals than the PRH-1 strain. On an overall average basis, considering all the soils, strain PRH-1 showed a greater reduction in number (54.8%) as compared to B-48 (48%). Giller et al. (1993) monitored the population of indigenous *R. leguminosarum* bv. *trifolii*, which remained at around 4×10^4 cells g^{-1} soil in FYM-treated soils (initial inoculum being $>10^9$ cells) but decreased by more than 60% in metal-contaminated soil after 150 d and by almost 95% after 840 and 900 d. A similar reduction in the survival and nodulating ability of indigenous and inoculated *R. leguminosarum* bv. *trifolii* in sterilized and unsterilized soil treated with sewage sludge was reported elsewhere (Purchase and Miles, 2001).

In contrast to this, Broos et al. (2004; 2005) examined soils treated with metal salts or sewage sludge 10 years ago and inoculated with *R. leguminosarum* bv. *trifolii* (10^8 cells g^{-1}), then incubated for up to 6 months, and concluded that Zn rather than Cd is the metal that is most toxic to free-living rhizobia. A significant decrease was only observed after 7 d and the maximum effect was observed after 28 d of incubation.

Heavy metals in soils are continuously accumulated by irrigation with sewage water. The heavy metal contents and particularly high Cd, Cu and Zn contents in the soil resulted in a decrease in the number of colony-forming units of inoculant strains from pea and Egyptian clover. Though some significant correlations were observed for survival, heavy metals and the physiological properties of the soils, more conclusive results could have been obtained with graded levels, preferably with large variations in the heavy metal contents and greater sample size. Increasing urbanization has resulted in a shift in sewage irrigation sites. Therefore, soil samples with the very high levels of heavy metals which earlier workers have reported could not be obtained. Further statutory limits for each metal should be established under Indian conditions, with respect to soil type, soil pH and cropping system.

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TIME-SAVING APPLICATION FOR SEQUENTIAL EXTRACTION OF HEAVY METALS BY OPTIMIZED BCR METHOD AND LIXIVIATION FROM UNTREATED SEWAGE SLUDGE

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This article describes an improvement in the modified BCR three-step sequential extraction procedure employed for heavy metals (Cd, Cr, Cu, Ni, Pb and Zn) in untreated domestic waste-water sewage sludge collected from different cities in Pakistan.

The BCR sequential extraction protocol requires 16 h for each step, whereas in the present work optimum recoveries of all heavy metals were attained in 10 h/step. The validity of the proposed BCR sequential extraction procedure was checked using certified reference material (BCR 483). Various parameters such as time interval (2–16 h) and sample mass (0.2–1.0 g) were studied to achieve optimum recovery of the heavy metals studied. The extracted analytes were determined by atomic absorption spectrometry. The optimum recovery of heavy metals from the certified reference material and from experimental samples was achieved at a sample mass of 0.4 g, while the time required for extraction on a mechanical shaker at 30 rpm was found to be 26–32 h. The sequence of easily available (acid-exchangeable) heavy metals was determined as Cd<Zn<Ni<Cr<Pb<Cu. With the exception of Cd the dominant fractions of the heavy metals were associated with organic matter, while 31.0 and 47.8% of Cd was present in acid-soluble and reducible forms, respectively. A lixiviation test (DIN 38414-S4) was used to evaluate the leaching of heavy metals from the domestic waste-water sewage sludge used for agricultural purposes.

Key words: untreated sewage sludge, domestic wastes, heavy metals, optimized BCR sequential extraction, leaching procedure, atomic absorption spectrometry

Introduction

Biosolid application to soils has undergone great development in recent years on account of its high nutrient (N and P) and organic matter content, which may improve both the chemical and physical properties of soil (Tsadilas et al., 1995; Danteravanich and Siriwong, 1998).

The increasing cost of landfill disposal and the phasing out of other environmentally unacceptable disposal options, such as ocean disposal, are other factors encouraging the increased use of biosolids in agriculture.

The application of solid waste-water sewage sludge to agricultural land is becoming increasingly popular because of the many benefits it can offer in terms of improved soil fertility and productivity, while land application also offers an economically viable disposal option (Johansson et al., 1999; Mosquera-Losada et al., 2001).

In addition to essential nutrients, solid sewage sludge also contains heavy metals. Although some metals are essential for plant growth, at higher levels they become toxic to plants, animals and humans (Taiz and Zeiger, 1998; Raskin et al., 1997). Heavy metals may accumulate in soil and plants when sludge is applied as fertilizer and may eventually produce harmful, unwanted environmental impacts such as phyto- and microbial toxicity, and contamination of the food chain and groundwater (Bhogal et al., 2003; Chaudri et al., 2000). When sewage sludge is used as a soil conditioner, toxic metals may limit the application rate. Literature data show that more than 50% of the sludges are unsuitable for use in agricultural areas due to their metal content (Couillard and Mercier, 1991). Metal contamination in soils amended with untreated sewage sludge has been repeatedly reported in the literature (Vooneburg and Veen, 1993). The remediation of soils contaminated with toxic metals is a challenging task because metals do not degrade with time (Wade et al., 1993).

It is therefore critical to know the physical and chemical properties of biosolids applied to land, particularly their elemental contents. Total metal concentration is a good indicator of the degree and extent of contamination, but in most cases provides limited information on the mobility and availability of heavy metals (Kazi et al., 2005; Jamali et al., 2009).

The composition of the sewage sludge depends to a large extent on the origin of the residues treated (i.e. waste water from domestic or industrial activities) (Jamali et al., 2006; Kazi et al., 2006).

Metal ions in biosolids are partitioned between the different phases present, i.e. organic matter, oxyhydroxides of iron, aluminium and manganese, phyllosilicate minerals, carbonates and sulphides (Ure and Davidson, 2001).

The determination of total trace metal content is not sufficient to assess the environmental impact of treated and untreated sewage sludge, because it is the chemical form of the metal in the sludge that determines its behaviour in the environment and its mobilization capacity (Filgueiras et al., 2002; Batstone and Keller, 2001). Toxic metal speciation studies continue to be of great value in environmental monitoring. This is because not only the concentration of these metals but more critically their existing forms in the environment will decide their toxicity, mobility and bioavailability. Single and sequential extraction schemes were developed in the early 1980s with the aim of assessing trace element bioavailability to plants and studying the environmental accessibility of trace metals (Quevauviller, 1998; Rauret et al., 2000; van Hullebusch et al., 2005). Many different schemes were developed over the past 20 years, as illustrated by the huge number of publications in the international scientific

literature. The Community Bureau of References (BCR) (now the Standards, Measurements and Testing Programme) proposed a modified three-step extraction procedure which was extensively studied to determine the different forms of elements in environmental samples (soil, sediment and sewage sludge) (Margui et al., 2004).

The uncontrolled disposal of sludge on agricultural lands near cities, and river and marine disposal are common practice in underdeveloped countries, because of the expense of providing proper treatment of the ever increasing amount of sewage sludge. In Pakistan up to 80% of untreated waste-water sewage sludge is used on agricultural lands near cities, where vegetables and grain crops are mostly grown (Kazi and Kazi, 2001; Kazi et al., 1999).

One of the main limitations of the BCR sequential extraction procedure is that it is extremely time-consuming. The main aim and objective of the present study was to improve the modified BCR procedure by reducing the shaking time on the mechanical shaker for each step when investigating the leaching of heavy metals from untreated domestic waste-water sewage sludge (DWS), which is used as agricultural fertilizer. The analytical methodology was assessed using a reference material (BCR 483). Sample masses between 0.1 and 1.0 g were tested. Furthermore, in order to gain additional information about the more easily mobilized metal forms in domestic sewage sludge, a leaching test based on the German Standard Method was developed to assess the leaching of heavy metals from DWS. Single extraction procedures are widely used as a general tool to evaluate metal pollution in environmental samples (Hardaway et al., 1999; Vander Sloot et al., 1996).

Materials and methods

Reagents and glassware

Ultra-pure water obtained from a Milli-Q purifier system (Millipore Corp., Bedford, MA, USA) was used throughout the work. The extractant solutions listed in Table 1 were prepared from analytical grade reagents (Merck, Darmstadt, Germany) and were checked for possible trace metal contamination. Ammonium acetate was purchased from Sigma Aldrich Co. Ltd. The acetic acid (glacial 100%), hydrochloric acid (65%, sp. gr. 1.4), nitric acid (37% sp. gr. 1.19) and hydrogen peroxide (30%) were of analytical reagent grade (Merck). Standard solutions of Cd, Cr, Cu, Ni, Pb and Zn were prepared by dilution of certified standard solutions (1000 $\mu g\ ml^{-1}$ Fluka Kamica) of the corresponding metal ions. Hydroxylammonium chloride of analytical grade (Merck, Poole, UK) was prepared prior to use. The certified reference material BCR 483 was purchased from the Bureau of References of the European Community (Brussels, Belgium). All glassware and plastic material was treated for 24 h in 5N supra-pure nitric acid prior to uses, and rinsed first with double-distilled water and then with ultra-pure water. Acid-washed polyethylene tubes (25 and 50 ml) were used for extraction.

Apparatus

A WTW pH meter was employed for pH adjustments of the reagents and the pH determination of the sewage sludge. Electrical conductivity was measured in a saturation extract of DWS using an EC meter (WTW InoLab Cond 720, Germany). Total nitrogen was determined

using Kjeldahl apparatus (Buchi Digestion Unit K-424, Switzerland). Metals were determined in a procedure involving sequential extractants and aqua regia digests using an Atomic Absorption Spectrophotometer (Hitachi Ltd., Model 180-50, S.N.5721-2) with a deuterium lamp back-ground corrector, equipped with a 10 cm burner head and graphite furnace GA-3, with hollow Hitachi, cathode lamps, and a Hitachi Model 056 recorder was used for recording the analytical data. Cu and Zn were measured under optimized operating conditions by FAAS with an air-acetylene flame, while Cd, Cr, Ni and Pb were determined by ETAAS. The measurement conditions for all elements are shown in Table 2.

Sampling and pre-treatment of sewage sludge

The sewage sludge samples were collected from the domestic catchment areas of various cities in Pakistan, at sites where the wastewater is separated from solid waste. These solid wastes are mostly used on agricultural land near the cities, where vegetables and grain crops are grown. Sampling was performed randomly every fifteen days in 2005 and 2006. Twenty-four composite samples of sewage sludge were made from more than 50 sub-samples (collected from each site). In the laboratory, all the sewage sludge samples were mixed together to make representative samples, which were spread on plastic trays in fume cupboards and allowed to dry at ambient temperature. After air drying for 8 days, the representative samples were ground in a centrifugal ball mill in order to homogenize them, passed through a 125-μm nylon fibre sieve and kept in polypropylene containers at ambient temperature before analysis.

Table 1
Chemical reagents and analytical conditions for the extraction method, followed by aqua regia digestion

Fraction	Extracting agent	Extraction time (h)*
Exchangeable and acid-soluble	20 ml CH_3COOH (0.11 M, pH = 7)	10
Reducible	20 ml NH_2OH–HCl (0.5 M, pH =1.5)	10
Oxidizable	5 ml H_2O_2 (30%, pH=2), shaking 1 h; 5 ml H_2O_2 (30%, pH = 2), shaking 1 h; heat to 85°C for 1 h, add 5 ml H_2O_2 (30%), shaking 1 h; heat to 85°C for 1 h, then add 25 ml CH_3COONH_4 (1 M, pH = 2)	
Residual	8 ml aqua regia (HCl/HNO_3, 3:1)	30 min^

* Shaking was applied at 30 rpm; ^ Digestion of the residual fraction in a microwave oven

Table 2a
Measurement conditions for ETAAS

	Pb	Cd	Ni	Cr
Lamp current (mA)	7.5	7.5	10	7.5
Wave length (nm)	283.3	228.8	232.0	357.9
Slit width (nm)	1.3	1.3	0.2	1.3
Cuvette	Cup	Cup	Tube	Tube
Carrier gas (ml/min)	200	200	200	200
Sample volume (μl)	10	10	10	10
Temperature programme				
Dry	80–120/15*	80–120/15*	80–120/15*	80–120/15*
Ash	300–600/15	300–600/15	500–700/15	300–700/15
Atomization	2000–2100/5	1500-1800/5	2500–2600/5	2600–2700/5
Cleaning	2100–2400/2	1800–2000/2	2600–2800/2	2700–2900/2

* Temperature range/time (sec)

Table 2b
Measurement conditions for AAS

Elements	Wavelength (nm)	Slit width (nm)	Lamp current (mA)	Burner height (mm)	Oxidant (air) kg/cm^2	Fuel (acetylene) kg/cm^2
Zn	213.8	1.3	10	7.5	1.60	0.2
Cu	324.8	1.3	7.5	7.5	1.60	0.3

Physico-chemical parameters

All the physico-chemical parameters, i.e. pH, conductivity, organic matter, total Kjeldahl nitrogen (TKN), sulphur, phosphate and silica, were recorded for each batch of sewage sludge samples using standard methods. The pH values of the 24 composite samples were determined using a 1:2.5 ratio of waste-water sludge : Milli Q water (McLaren and Clucas, 2001). Triplicate samples of each DWS were burned in a muffle furnace at 550°C for four hours to determine the organic matter content, which was then calculated gravimetrically based on the weight difference (Preer et al., 1980) and organic carbon was determined by the Walkley-Black method (Soltner, 1988). The total sulphate-sulphur was determined using the methods proposed by Reisenauer et al. (1973) and Kazi and Katz, (1990). Phosphorus was determined as proposed by Gibson et al (1976). Total Kjeldahl-N was determined according to Bruemmer and Mulvaney (1982). All results are the means of three independent determinations for each composite sample (n=72), as summarized in Table 3.

Sequential extraction procedure

The modified BCR sequentional extraction procedure (Pueyo et al., 2001) was applied to air-dried triplicate samples of each composite sample of DWS and six replicates of the certified reference material BCR 483. In order to determine the optimum sample size to be analysed, model experiments were performed with various sample quantities (0.2–1.0 g). To optimize the BCR method, the weight of the samples and volume of extractants were reduced, while the ratio of solid samples to the volume of extraction solution was the same. All the extractions were performed by shaking in a mechanical, end-over-end shaker at a speed of 30 rpm at room temperature for various time intervals (6–16 h). The details of the experiment protocol are available elsewhere (Kazi et al., 2005; Sahuquillo et al., 1999).

To correct the dry mass of the air-dried sewage sludge samples, triplicate samples of each batch of air-dried samples of DWS and BCR 483 were dried in an oven at 100±5°C to constant mass. From this a "dry mass correction" was obtained, which was applied to all the analytical values reported (i.e. the results are given as quantity of metal μg g^{-1} dry weight). Blank extractions (without sample) were subjected to the complete procedure for each set of analyses.

Table 3
Physico-chemical parameters of domestic sewage sludge

Parameters	Mean ±S.D
pH	7.2±0.41
Silica %	51.1±2.4
Dry matter %	87.6±3.1
Organic matter %	34.2±4.4
Total nitrogen, μg g^{-1}	8650±613
Total sulphur, μg g^{-1}	902±42.3
Total phosphate, μg g^{-1}	1120±85.9

Optimization of sequential method

Effect of shaking time

To optimize the rapid BCR sequential extraction method, six replicate air-dried 0.2–1.0 g samples of BCR 483 and two composite samples of DWS were placed in 25 or 50 ml polyethylene tubes (according to the volume of extracting reagents). These tubes were also used for centrifugation to minimize possible losses in the centrifuge washing steps. The volumes of extracting reagents were chosen to maintain the solid–liquid ratio used for the original modified BCR method. The extracting reagents were added in 1–3 steps, and shaken in an end-over-end mechanical shaker for different time intervals (6, 8, 10, 12, 14, 16 h) at room temperature (25–35°C), which was higher than that used in most other research (usually 20°C) (Lacal et al., 2003; Mossop and Davidson, 2003), due to the warm climate of Pakistan. To check the maximum shaking time for optimum recovery of the heavy metals, two tubes each of DWS and BCR 483 were removed after (2 h) centrifuged to separate the extractant from the residue. This was repeated every two hours to a maximum shaking time of 16 h. The residues obtained were subjected to the 2nd step of the BCR sequential extraction procedure, as described above.

Effect of sample size

Replicate samples of DWS and BCR 483 weighing 0.1, 0.2, 0.4, 0.8 and 1.0 g were placed in 25 or 50 ml tubes (according to the solid–extractant ratio) and extractants were added in quantities of 4, 8, 16, 32 and 40 ml, respectively. All the tubes were subjected to mechanical shaking at 30 rpm. The residues obtained after the first step were treated as described in the modified BCR method.

After each extraction step for both optimized strategies (time intervals and sample mass) the supernatant liquid was separated from the solid phase by centrifugation at 3000 rpm for 10 min. After the final extraction step the extractants were evaporated to reduce the volume, made up to 25 ml with 2N nitric acid and stored at 4°C in polyethylene vessels (Bibby Sterilin Ltd., UK). Between each extraction step, the residues obtained were washed with 10 ml deionized water and shaken for 15 min. The water was discarded after centrifugation.

The maximum recovery of all the metals was obtained after 10 h, instead of the 16 h reported in the literature (Margui et al., 2004; Mossop and Davidson, 2003). A sample mass of 0.2 to 0.3 g gave maximum metal recovery, so for the rest of the extraction procedures a sample mass of 0.25 g and a shaking period of 10 h were used.

Pseudo-total and residual digestion

The pseudo-total metal contents of DWS and BCR 483 were determined by digestion with aqua regia (Anxiang et al., 2003). The residual materials remaining at the end of the third step of the sequential extraction method, 200 mg of duplicate oven dried samples of all twenty-four composite samples of DWS and five samples of BCR 483, were placed in PTFE flasks and treated with 65% HNO_3 (2 ml) and 6 ml of 37% HCl. The flasks were placed in a PTFE container and heated following a one-stage digestion programme (250 W, 30 min). After cooling, the sample digests were filtered through a membrane filter of pore size 0.45 μm, pre-washed with ultra-pure water, transferred to a 25 ml flask and brought up to volume with 2 N HNO_3.

Leaching tests

The leaching test provides information about the behaviour of a biosolid when it comes into contact with water. The experimental protocol followed the German Standard Method (DIN 38414-S4), which uses Milli Q water as an extractant. Duplicate 1.0 g air-dried samples of DWS (sieved <90 μm) were weighed into Pyrex flasks, then leached at room temperature for a period of 12 to 24 h using a 1:10 (biosolid: milli Q water) ratio. After the leaching period had elapsed, the undissolved residue was separated by filtration using a membrane filter of pore size 0.45 μm, and pre-washed with ultra-pure water. Finally, the pH value, electrical conductivity and Cd, Ni, Cu, Cr, Pb and Zn concentrations were measured (Table 4).

Table 4
Leachability (μg g^{-1}) by water (DIN 38414-S4) and contamination factor for each element in domestic waste-water sewage sludge

Leaching test		Contamination factor (C_f)*
pH	6.84–7.86	
Conductivity (mS cm^{-1})	985–2120	
Cadmium	1.37±0.23	11.6
Chromium	0.567±0.04	0.796
Copper	0.869±0.11	0.841
Nickel	0.970±0.12	1.75
Lead	0.638±0.06	0.734
Zinc	3.46±0.21	4.18

$$*C_f = \frac{\Sigma \text{ step } 1+2+3}{\text{Residual}}$$

Evaluation of analytical performance

The analytical performance of the laboratory procedure was evaluated by analysing a certified reference material, BCR 483. Six replicate samples were analysed for each step and the values (expressed in μg g^{-1}) were reported on a dry mass basis and compared with certified values of all the heavy metals studied (Rauret et al., 2000; Mossop and Davidson, 2003; Quevauviller, 2002).

For an internal check of the procedure, the residues from Step 3 were digested in aqua regia and the sum of all the steps (SEM) was compared with the pseudo-total (TEM) obtained by aqua regia digestion of separate samples of DWS and BCR (Tables 5 and 6). The recovery of the sequential extraction procedures was calculated as follows:

$$\text{Recovery (\%)} = \frac{\Sigma\text{SEM in the individual fractions}}{\text{TEM}} \times 100$$

Results and discussion

Sludge characteristics

The basic characteristics of the sewage sludge samples (pH, dry matter content, organic matter) are presented in Table 3. The mean pH values of 24 composite samples of DWS were found to be in the range of 6.84–7.86. The mobility and leaching of heavy metals increases at low pH (at 6.5 for instance) and decreases as the pH approaches neutral or rises above 7.0. Organic matter is an important component because it tends to form either soluble or insoluble complexes with trace and toxic metals, or it migrates or is retained in the soil. The considerable amount of total nitrogen, sulphur and phosphates in the sludge suggest it could have a beneficial impact when used as an agricultural fertilizer. The maximum concentration of Cu, Cr, Ni, Pb and Zn was found in the oxidisable fraction, while Cd was mainly released from the exchangeable and reducible fractions.

Sequential extraction

It is known that sequential chemical extraction techniques represent a rather arbitrary way of separating different metal species. This is because the

reagents used are not very selective towards one particular species, and rarely completely solubilize the species. Nevertheless, the sequential chemical extraction technique does provide information on the origin, the mode of occurrence, the biological and physicochemical availability, the mobilization and the transport of metals in sewage sludge.

It was not possible to quantify the metals in the extracts by direct calibration with standard solutions in 2 N HNO_3 due to the matrix effects of the different reagents on the extractants. To minimize these matrix effects and to prepare standards for different matrices, all the extractants were evaporated to semi-dryness and redissolved in 2 N nitric acid in order to obtain the same matrix for all samples and standards. Therefore, the same standards of each element were used for all elements in the extractants.

Optimization of the proposed modified BCR method

Effect of time period

To develop a time-saving strategy, the optimized BCR extraction method was applied on certified reference material (BCR 483) and on soil amended with sewage sludge. Shaking was carried out on a mechanical shaker at 30 rpm for different time intervals (6–16 h). The optimum recovery of the heavy metals was obtained after a time period of 8 h for Cd and Zn, while 10 h was required for the optimum recovery of Pb, Cu, Cr and Ni in all three BCR extraction steps.

The precision and accuracy of the proposed method was evaluated using BCR 483, which has certified values for the heavy metals studied (Table 5a, 5b). Precision in terms of relative standard deviation (RSD) % was < 10, while in the first step Cr and Pb had RSD % values of 29.4 and 33.3, respectively, which is lower than reported values (Rauret et al., 2000). The RSD % was calculated as:

$$\text{RSD}\ (\%) = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

A comparison was also made between the aqua regia results on the original sample (pseudo-total) and the values from the three steps plus residual (3 steps + aqua regia extractable from residual). As can be seen in Table 5b, no significant differences were observed between the pseudo-total metal content following the aqua regia digestion and the sum of the extracted metals following the sequential extraction procedure. Low relative errors (<10%) indicate the good quality of the results obtained.

Effect of sample mass

In the proposed method for the BCR sequential extraction of heavy metals, lower sample masses were used. Replicate samples were taken to make the results representative of the whole samples. To assess the effect of sample mass on the three-step sequential extraction of heavy metals following the BCR procedure, sample masses of 0.1–1.0 g of BCR 483 and DWS samples were tested. In each case the recommended BCR soil : extractant ratios were applied.

Table 5a

Indicative values for extractable heavy metal contents (μg g^{-1}) in BCR 483 following the BCR three-step sequential extraction scheme, and the aqua regia extractable contents

Extraction steps/Element	Conventional extraction		Indicative value	
	Mean/SD	RSD %	Mean/SD	RSD %
First step				
Cd	11.0±0.4	3.6	10.0±0.8	8.0
Cr	10.2±3.0	29.4	9.4±3.5	37.2
Cu	16.2±1.3	8.0	16.8±1.5	8.9
Ni	18.2±1.6	8.8	17.9±2.0	11.2
Pb	0.84±0.28	33.3	0.76±0.7	92.0
Zn	438.5±40.0	9.1	441.0±39.0	8.8
Second step				
Cd	24.5±2.0	8.2	24.8±2.3	9.3
Cr	660.0±85.0	12.9	654.0±108.0	16.5
Cu	148.5±16.5	11.1	141.0±20.0	14.2
Ni	25.0±2.5	10.0	24.4±3.3	13.6
Pb	381.0±20.0	5.2	379.0±21.0	5.5
Zn	452.0±56.0	12.4	438.0±56.0	12.8
Third step				
Cd	1.62±0.2	12.3	1.22±0.48	39.3
Cr	2231.0±280	12.6	2215.0±494.0	22.3
Cu	128.0±22.5	15.1	132.0±29.0	22.0
Ni	5.3±0.8	9.2	5.9±1.4	23.7
Pb	65.2±6.0	17.5	66.5±22.0	33.1
Zn	38.4±6.8	17.7	37.1±9.9	26.7
Aqua regia (residue)				
Cd	0.38±0.14	36.8	0.423±0.16	37.8
Cr	286.0±28.0	9.8	183.0±40.0	21.9
Cu	45.5±3.2	7.0	43.3±3.8	8.8
Ni	14.9±2.2	14.8	15.2±4.3	28.3
Pb	75.3±13.5	17.9	76.9±17	22.1
Zn	83.4±7.4	8.9	82.1±9.6	11.7

RSD: Relative standard deviation

Table 5b

Comparison of results (mean ± SD) obtained by using the conventional BCR extraction method and the aqua regia extraction protocol for BCR 483 (μg g^{-1})

Elements	Aqua regia (Pseudo-total)	Sum of 3 steps + Residual	Relative error (%)
Cd	36.3±2.5	37.5±2.05	3.3
Cr	3230±375	3187.2±293.97	–1.3
Cu	340.6±15.8	338.2±28.11	–0.7
Ni	64.8±6.1	63.4±3.78	–2.2
Pb	510±47.0	522.3±24.87	2.4
Zn	996.8±78.5	1012.3±69.55	1.6

$$\text{Relative error (\%)} = \frac{(\text{Sum of 3 steps + Residual}) - \text{Pseudo-total}}{\text{Pseudo-total}} \times 100$$

The amounts of heavy metals extracted at each stage of the procedure did not vary significantly for different sample masses. Overall recovery (Σ step 1+2+3+Residual) was 98–100% of the pseudo-total content of heavy metals. The precision of the heavy metal values obtained with a sample mass of 0.25 g had good RSD % as compared to the highest sample mass of 1.0 g (Rauret et al., 2000) (Table 5).

Comparison of three-stage sequential extraction of DWS

The optimized BCR sequential extraction procedure was applied to assess heavy metal fractionation in the samples. This extraction procedure consists of three extraction steps, namely: Step 1: extraction with acetic acid (0.11 M); Step 2: extraction with hydroxylamine hydrochloride (0.5 M, pH=1.5); Step 3: extraction with H_2O_2, 8.8 M (2×1 h, 85°C) followed by extraction with ammonium acetate (1.0 M). Additionally, a fourth step was added by dissolving the final residue in aqua regia. The results of speciation of heavy metals (Cd, Cu, Cr, Ni, Pb and Zn) in DWS samples are presented in Table 6.

Table 6a
Speciation of toxic metals in domestic waste-water sludge samples (μg g^{-1})

	Mean	SD	RSD (%)
First step			
Cd	6.51	0.62	9.52
Cr	1.92	0.14	7.29
Cu	1.99	0.13	6.55
Ni	3.47	0.27	7.79
Pb	2.95	0.18	6.09
Zn	21.2	1.7	8.00
Second step			
Cd	10.0	0.75	7.48
Cr	3.73	0.26	6.96
Cu	8.60	0.46	5.35
Ni	6.34	0.38	5.99
Pb	9.14	0.68	7.44
Zn	61.7	3.6	5.84
Third step			
Cd	2.79	0.19	6.82
Cr	21.5	1.2	5.57
Cu	26.4	1.9	7.20
Ni	19.3	1.1	5.71
Pb	36.2	2.3	6.35
Zn	122	6.9	5.64
Aqua regia (residue)			
Cd	1.67	0.12	7.18
Cr	34.2	2.9	8.48
Cu	43.9	3.5	7.97
Ni	16.7	1.4	8.40
Pb	65.8	3.8	5.77
Zn	49.0	3.1	6.32

SD: standard deviation; RSD: Relative standard deviation

Table 6b
Comparison of results (mean ±SD) obtained from sequential and aqua regia extraction of domestic sewage sludge (μg g^{-1})

Elements	Aqua regia (pseudo-total)	Sum of 3 steps + Residual	Relative error (%)
Cd	20.6±1.8	21.0±1.8	1.95
Cr	62.5±3.6	61.4±3.8	–1.77
Cu	82.6±4.8	80.9±4.7	–2.06
Ni	45.9±3.5	45.7±3.6	–0.330
Pb	115±8.7	114±7.8	–0.773
Zn	252±12.5	254±13.5	0.870

Acid-soluble fraction

The exchangeable and acid-soluble fractions show the amount of each element that would be released by the anaerobic sludge if the conditions became slightly acidic (pH < 7.0). This phase is susceptible to changes in pH, generally being targeted with a mild acid (Tyagi et al., 1997; Ravishankar et al., 1994). In the present work 0.11 M acetic acid was used to measure the acid-soluble fraction of heavy metals. This reagent is capable of dissolving carbonates without a significant attack on organic matter, Fe and Mn oxides and aluminosilicates. The exchangeable fraction (Step 1: CH_3COOH, 0.11 M) may indicate the easily available heavy metals for plant uptake and release into the environment.

The acid-soluble fraction of the heavy metals increased in the order Cu<Pb<Cr<Ni<Zn<Cd, with values of 2.45, 2.59, 3.13, 7.58, 8.36 and 31.0% of the pseudo-total content of these heavy metals, as shown in Figure 1. It was observed that the toxic metal Cd is present in DWS in a more available form, so the use of DWS on agricultural land could have a potentially hazardous effect on the environment. The other heavy metals were also present in high amounts as compared to the values found in the literature, especially Cr. This could be due to the presence of small tanneries and other local industries in the collection areas (Li et al., 2001).

Reducible fraction

This fraction theoretically represents the contents of metals bound to iron and manganese oxides, which are released if the matrix is subjected to reductive conditions.

However, iron and manganese oxides are relatively scarce in sewage sludge. Samples with large proportions of this fraction are found in soil and sediments. Hydroxylamine hydrochloride in a nitric acid medium is the most widely used reagent for leaching the easily reducible fraction.

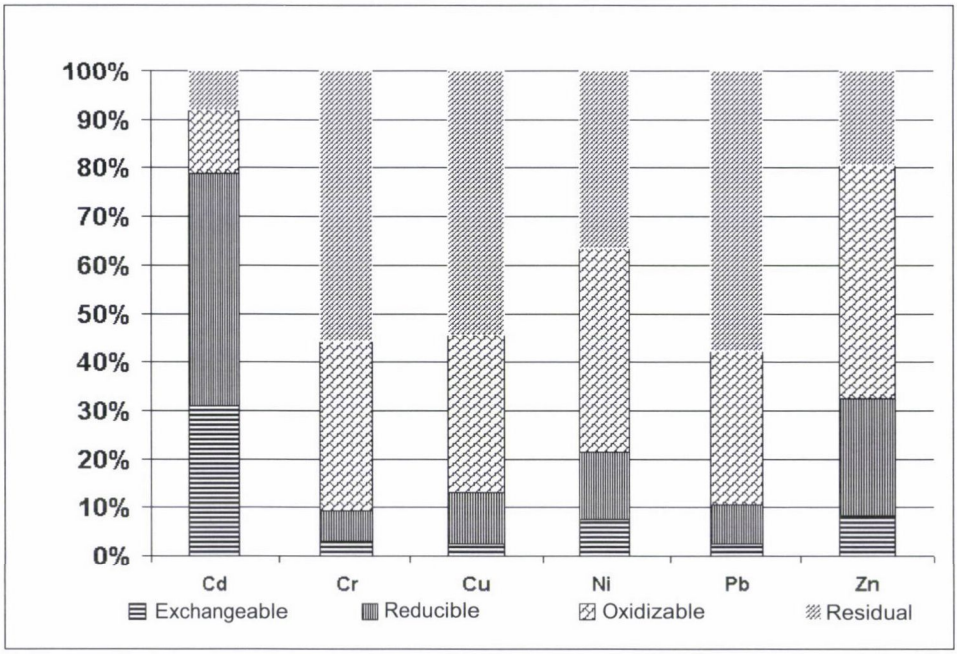

Fig. 1. Heavy metal partitioning by conventional extraction in domestic waste-water sewage sludge

In this optimized BCR procedure, a high concentration of hydroxylamine hydrochloride (0.5 M) at low pH (1.5) removed high levels of heavy metals. In the DWS samples studied this was the predominant fraction for cadmium (47.8% of the pseudo-total content). These results are consistent with other studies (Jaradat, 2002). Heavy metals that are retained in this form may be released from sewage waste or soil if there is a change in the oxidation state of Fe and Mn, and could be a long-term source of contamination (Wasay et al., 2001).

Oxidizable fraction

The third fraction releases the organic matter in the DWS. The organic fraction released under oxidizing conditions is not considered to be mobile and the bioavailable metals are incorporated into stable, high-molecular-weight humic substances, which release small amounts of metals over long time periods.

When sewage sludge is used as a soil conditioner, the toxic metals associated with the oxidizable phase are assumed to remain in the soil for long periods. Degradation of organic matter under oxidizing conditions can lead to the release of the metals bound to these organic components (Davidson et al., 1999). The toxic metals bound to organic matter and sulphides can easily be released under oxidizing conditions, so an oxidation process was used to leach the metals associated with this phase. Organic substances exhibit a high degree of selectivity for divalent ions compared to monovalent ions. In the present work, the most common oxidant, hydrogen peroxide, was used in an acid medium (Stone and Marsalek, 1996).

In general, hydrogen peroxide is applied to a heated medium (85°C) for several hours to dissolve the organic matter, which is a compromise between strongly attacking the organic matter and minimizing any alterations to silicates. However, H_2O_2 heated to 85°C is still a formidable oxidant of organic matter.

The oxidizable fraction was dominant for all the heavy metals except cadmium in all batches of DWS, increasing in the order Cd<Pb<Cu<Cr<Ni<Zn, with values of 13.3, 31.7, 32.6, 35.1, 42.1 and 48.1%, respectively.

Residual fraction

The heavy metals associated with the crystal lattice of minerals and in well-crystallized oxides are mostly non extractable. In this work the residual fraction made up 57.7, 55.7, 54.3, 36.4, 19.3 and 7.96% of the total Pb, Cr, Cu, Ni, Zn and Cd extracted. These results show that a high concentration of Cr, Cu and Ni is present in DWS as sulphides, which are refractory to H_2O_2 oxidation.

Leaching test DIN 38414-S4

The leaching test revealed the amount of heavy metals leached out at neutral pH, i.e. the pH of water. This form of heavy metals is very important for the environment. The extraction procedure was performed with Milli-Q water based on the German Standard Method DIN 38414-S4. Each composite sample of DWS was extracted with deionized water at room temperature. In each individual extract, pH and conductivity were measured and the heavy metals were determined by atomic absorption spectrometry. The conductivity of the water extract in DWS samples ranged from 985–2120 mS cm^{-1}, which is within the statutory limits reported by Margui et al. (2004). The results of leachability with water are shown in Table 4.

Conclusions

The optimized BCR three-step sequential extraction procedure for predicting the fractionation of heavy metals (Cd, Cr, Cu, Ni, Pd and Zn) from domestic waster-water sewage sludge was modified to reduce the time of extraction for each step. The proposed method was validated with a certified reference material, BCR 483, having the same matrixes as the experimental samples. The overall extraction time was reduced from 51 h to 32 h. The effect of sample mass was also studied, and the recovery of heavy metals was found to be independent of sample mass.

A significant proportion of the heavy metals, especially Cd, was present as exchangeable and acid-soluble species in the DWS samples. The predominance of the oxidizable fraction was observed for all the heavy metals except Cd, which was extracted mostly in the first two fractions. A leaching test was also applied to study the easily mobilized forms of heavy metals in DWS. This test provides preliminary information on the behaviour of waste when it comes into contact with water. The results of the lixiviation test showed that untreated samples of wastes contain highly mobile Cd, while Ni and Cr was also leached by water.

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ASSIMILATION OF VARIOUS ORGANIC CARBON SOURCES BY *HAEMATOCOCCUS* STRAINS

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Differences in the assimilation of individual organic compounds (5 mM sugars and L-asparagine) under mixotrophic growth conditions were described for three naturally occurring *Haematococcus* strains.

The effects of assimilation were measured by the growth intensity and size of algal cells, and the effect of colour changes in the cultures was observed. Some compounds caused the cell colouration to change from green to yellow, being the result of chlorophyll disappearance and the accumulation of yellow secondary carotenoids. In the present experiment none of the cultures turned red, thus excluding the intense accumulation of the commercially interesting carotenoid, astaxanthin.

Key words: astaxanthin, green algae, *Haematococcus*, secondary carotenoids, sugars

Introduction

The freshwater microalga *Haematococcus*, which belongs to the *Volvoccales*, is a natural source of the red ketocarotenoid astaxanthin (3,3'-dihydroxy-4,4'-dioxo-β-carotene). This pigment is formed in large quantities under unfavourable environmental conditions, such as a lack of necessary nutrients (nitrogen starvation, intense irradiation or a combination of these two factors). The cysts, aplanospores, formed under these conditions are red and contain a large amount of astaxanthin (Lee and Lababpour, 2006; Margalith, 1999; Montsant et al., 2001).

There are two reasons for the huge interest in astaxanthin in recent years. It was found that astaxanthin is a strong antioxidant, which possesses antiphlogistic, antineoplastic, antidiabetic and immunomodulating activity (Guerin et al., 2003). Antibacterial activity, especially in relation to the bacterium *Helicobacter pylori* (Lee et al., 2003; Hussein et al., 2005; Jyonouchi et al., 2000; Zhang et al., 1999), was also confirmed for astaxanthin.

Investigations on these biomedical activities led recently to an intensified search for a way to improve astaxanthin biosynthesis and to find factors which may favour this process.

Only a small number of lower plants are rich natural sources of astaxanthin. These include *Haematococcus* strains and the yeast species *Phaffia rhodozyma*. However, the astaxanthin formed by the microalga *Haematococcus* is better assimilated by animal organisms (Margalith, 1999). Some bacteria also possess the ability to form this ketocarotenoid, e.g. *Brevibacterium*, *Mycobacterium* and *Agrobacterium* (Dominguez-Bocanegra et al., 2004).

It has recently been shown that some organic compounds could have an influence on the growth and accumulation of astaxanthin, e.g. L-asparagine increases the growth of *Haematococcus*, while malonic acid and acetic acid stimulate the accumulation of astaxanthin (Orosa et al., 2005; Tripathi et al., 1999). Vinblastin, a cytostatic compound used in oncology, increases the biosynthesis of astaxanthin and inhibits the growth of this microalga (Margalith, 1999). A search was thus begun for substances interfering with astaxanthin biosynthesis.

In the present work the ability of three algal strains of *Haematococcus* to assimilate selected single sugars under mixotrophic growth conditions was investigated. Changes caused by these substances in the profile of cell pigmentation were also monitored.

The criterion used to measure the assimilation of these compounds was the number of cells in medium containing the individual sugars after the incubation period, together with cell size, recorded as the diameter and estimated volume of the cells. The influence of individual sugars on the accumulation of astaxanthin was also examined.

Materials and methods

Haematococcus investigations were carried out on three algal strains designated as Re (isolated near Potsdam in the locality of Rehbrücke, Germany), Er (isolated in Cieszyn, Silesia, Poland) and St (isolated in Stramberk, Morava, Czech Republic), all provided by Prof. J. Burczyk. All the strains were axenic and bacteriologically clear.

The algae were maintained and cultivated on the nutrient medium described by Kessler and Czygan (1970), as modified for microalgal growth by Burczyk (1982). The preliminary cultivation of the strains was carried out in 1 dm^3 Erlenmeyer flasks under sterile conditions for two weeks. Subsequently, algal suspensions of each *Haematococcus* strain were sedimented overnight, then aseptically decanted and centrifuged (800 g, 7 min). After washing with distilled water the cells were resuspended in about 200 ml of water and left for five days in Erlenmeyer flasks for starvation. A suspension with a density of 1.5 million of cells per 1 cm^3 of culture was used as inoculum.

In the assay on the assimilation of individual sugars as a main source of carbon, a modified nutrient medium OHM (Optimal Haematococcus Medium) was used (Fabregas et al., 2000). The following sugars were tested: L-arabinose, L-fucose, D,L-xylose, L-rhamnose, D-ribose, D-fructose, D-galactose, D-glucose, D-mannose, D-glucosamine, lactose and sucrose at a final concentration of 5 mM/dm^3. The experiment was carried out under mixotrophic conditions in test-tubes 12 mm in diameter.

The same OHM medium was used for the experiment showing changes in the colour of the culture as a function of the D-glucose and sucrose concentration. These sugars were added to the medium in a wide range of concentrations from 5 to 50 mM. The experiment was carried out under mixotrophic conditions in 120 mm test-tubes 12 mm in diameter, as previously.

The individual sugars were dissolved in OHM I nutrient medium at a concentration of 5 mM and aliquots of 5 cm^3 were placed in test-tubes, closed with cotton-wool corks, covered with aluminium foil and sterilized at a temperature of 117°C for 20 min. A 0.2 cm^3 quantity of inoculum (density about 1.5 million cells/cm^3) of the algal strains was added to each test-tube using a Pipetting Syringe (Rocofix, Georg and Henke GmbH). For each strain there were three repetitions of each sugar at each concentration. Additionally 5 test-tubes were filled with OHM medium without sugars, as a control. The test tubes were placed in a slanting position at an angle of 30° in a thermostat at 25±1°C, under illumination by fluorescent lamps (4000 lux), with a 16/8 h light/dark photoperiod. The test tubes were stirred by hand twice a day and cultivated for 30 days under the same conditions.

The growth of the algal cultures was checked by determining the cell number. A 1 cm^3 cell suspension was taken aseptically from each variant and the number of cells was determined microscopically using a Bürker's hemocytometer. The size of the cells was also measured using an Optiplot-2 Microscope (Nikon), connected through on RGB camera (Cohu) to a personal computer (software: Lucia G4.51, Laboratory Imaging). The number and size of cells make it possible to estimate the biomass produced during the cultivation period.

Results

Table 1 shows the number of cells obtained after cultivating algal strains of *Haematococcus* sp. on OHM medium supplemented with 5 mM of individual sugars, while Table 2 presents the cell size classes and the proportion of each class in the total number of cells.

In general strains Re and St exhibited a qualitative similarity in the assimilation of sugars. Relatively good assimilation was observed on medium containing D-mannose, D,L-xylose, D-fructose, L-arabinose, D-glucose, D-glucosamine and sucrose. The weakest growth of *Haematococcus* sp. strain Re was observed on medium with L-fucose, but this sugar was well assimilated by *Haematococcus* sp. St. However, there were certain differences in cell size between the strains. *Haematococcus sp.* strain St was characterized by relatively small cell size with the majority of cells in classes I–III, and none in class V. Strain Re formed bigger cells, categorized mainly in classes II and III, while classes IV and V were also relatively frequent in the case of supplementation with disaccharides.

The *Haematococcus sp.* strain Er, on the other hand, showed comparatively weak assimilation of the applied sugars. The most intensive growth occurred on medium with L-arabinose and the weakest in the presence of D-glucosamine as the main carbon source. This strain formed relatively small cells in classes II and III, though in some cases larger cells (classes IV and V) were also observed.

The *Haematococcus sp.* strain Re had the least tendency to change colour during 30 days of cultivation, while strain St formed yellow-orange cultures during the same period in almost every combination of added sugars. The only exception was when the medium was supplemented with D-glucosamine, where no change of colour occurred.

Table 1
Comparison of culture density of *Haematococcus* strains grown mixotrophically on modified OHM nutrient medium supplemented with 5 mM sugars

Compounds	Strains (million/cm^3)		
	Re	Er	St
L-Arabinose	2.52 ± 0.04	1.19 ± 0.07	1.99 ± 0.03
L-Fucose	0.89 ± 0.03	0.41 ± 0.02	2.15 ± 0.03
D.L-Xylose	2.43 ± 0.16	0.23 ± 0.02	2.13 ± 0.02
L-Rhamnose	1.52 ± 0.05	0.49 ± 0.01	1.28 ± 0.03
L-Ribose	1.35 ± 0.02	0.47 ± 0.02	1.46 ± 0.04
D-Fructose	2.52 ± 0.05	0.96 ± 0.05	1.38 ± 0.06
D-Galactose	1.03 ± 0.04	1.05 ± 0.01	1.54 ± 0.02
D-Glucose	2.42 ± 0.17	1.04 ± 0.06	2.15 ± 0.04
D-Glucosamine	2.45 ± 0.04	0.16 ± 0.02	1.12 ± 0.03
D-Mannose	2.78 ± 0.06	0.76 ± 0.02	2.05 ± 0.07
Sucrose	2.27 ± 0.05	1.04 ± 0.07	1.36 ± 0.09
Lactose	1.61 ± 0.09	0.99 ± 0.04	1.86 ± 0.07
Control	3.34 ± 0.10	1.03 ± 0.04	1.63 ± 0.03

Table 2
Proportion of each size class in the total number of cells of *Haematococcus* strains grown on modified OHM medium with the addition of 5mM concentrations of various sugars

Strains	*Haematococcus* sp. Re					*Haematococcus* sp. Er					*Haematococcus* sp. St			
Class	I	II	III	IV	V	I	II	III	IV	V	I	II	III	IV
Size (μm^2)	<20	20–50	50–100	100–150	150–200	<20	20–50	50–100	100–150	150–200	<20	20–50	50–100	100–150
L-arabinose	0	60	40	0	0	0	30	55	10	5	0	65	35	0
L-fucose	5	60	35	0	0	10	15	35	30	0	35	65	0	0
D,L-xylose	0	35	65	0	0	0	40	50	10	0	25	75	0	0
L-rhamnose	0	80	20	0	0	0	70	30	0	0	40	40	20	0
L-ribose	25	45	30	0	0	0	50	45	5	0	30	50	20	0
D-fructose	0	80	20	0	0	20	20	50	10	0	0	60	40	0
D-galactose	0	70	20	10	0	0	60	25	5	10	10	60	30	0
D-glucose	0	75	25	0	0	0	40	60	0	0	15	55	25	5
D-mannose	0	70	30	0	0	0	30	30	20	20	20	70	10	0
D-glucosamine	40	60	0	0	0	0	60	35	0	5	15	65	20	0
Sucrose	0	0	55	25	20	40	20	40	0	0	30	50	20	0
Lactose	0	25	50	15	10	0	70	30	0	0	30	30	40	0
Control	0	70	30	0	0	0	15	50	25	10	20	40	30	10

The additional experiment (data not shown) concerning the change in culture colour as a function of D-glucose and sucrose showed that *Haematococcus* sp. strains Er and St reacted to an increasing concentration of sucrose by a decrease in the intensity of green colour and the cells turned yellow. In the case of *Haematococcus* sp. strain Re, no change of colour was observed during the whole period of cultivation on medium supplemented with sucrose and the culture remained light green over the whole range of sucrose

concentration. However, when the strain Re was cultivated in the presence of D-glucose in the range 5–50 mM it exhibited an intensive yellow-orange colour, but did not turn red.

Cell aggregation was also taken into consideration and it was observed that the highest tendency to form aggregates was exhibited by *Haematococcus* sp. strain Re (data not shown) and the lowest by the strain St. Differences in the assimilation of the tested sugars gave evidence of strain differentiation in nature. These differences could be genetically determined.

Discussion

The influence of individual organic compounds, generally sugars, on the accumulation of secondary carotenoids in *Haematococcus* strains varies. Their intense accumulation by many green algae is correlated with a change in the colour of the cells from green to orange or red. The change in the colour of *Haematococcus* cells to intense red under unfavourable environmental conditions is directly connected with the accumulation of astaxanthin. In the present assay no red colour was observed, so the intensive production of astaxanthin under the applied conditions can be excluded.

Haematococcus accumulates astaxanthin when exposed to disadvantageous environmental factors, such as nitrogen starvation, strong illumination, high values of temperature, pH or salt concentration and phosphorus deficit (Fabregas et al., 2003; Kang et al., 2005) and forms thick-walled cells impregnated with algaenan, an acetolysis-resistant biopolymer (Montsant et al., 2001). The addition of sodium acetate, L-asparagine, trace elements and vitamin B causes an increase in biomass production and total astaxanthin production during a short time under both autotrophic and mixotrophic conditions (Tripathi et al., 1999). For *Haematococcus,* thiamine (vitamin B_1) plays the role of a growth factor, while vitamin B_{12} stimulates growth but it is not a necessary growth factor (Fabregas et al., 2000). The action of these components depends on their concentration. For example, sodium acetate promotes the growth and synthesis of secondary carotenoids at low concentrations, but high concentrations of acetate effectively inhibit growth and cause an increase in astaxanthin accumulation in *Haematococcus* cells (Margalith, 1999). Similar effects are induced by malonate, though the accumulation of astaxanthin is higher than in the case of acetate.

Sodium bicarbonate is also a good carbon source for microalgae. The synthesis of astaxanthin is more effective in the presence of sodium bicarbonate compared with acetate at the same level of illumination (Schoefs et al., 2001).

Another factor stimulating astaxanthin accumulation in *Haematococcus* is intensive illumination (345 μmol photon m^{-2} s^{-1}) (Lee and Lababpour, 2006), but if the illumination is too strong it causes cell damage (Harker et al., 1996). Grünewald et al. (2000) showed that light is necessary for astaxanthin

production. However, *Haematococcus pluvialis* has the ability to grow under heterotrophic conditions in the presence of sodium acetate even in darkness, though under such conditions only small quantities of astaxanthin are produced, mainly as monoesters (Zhang et al., 1999). The production of astaxanthin under photosynthetic conditions is much greater than under heterotrophic conditions at the same illumination (Schoefs et al., 2001).

The role of nitrogen starvation and its relation to astaxanthin production is not yet fully explained. Some differences were described between algae belonging to the *Chlorococcales* and *Volvocales* (Burczyk, 1982). However, nitrogen plays an important role in cell division and secondary carotenoids production as well being the necessary for protein synthesis. It may also be involved in astaxanthin production by *Haematococcus* (Orosa et al., 2005). Generally, nitrogen starvation increased the astaxanthin accumulation by *Haematococcus*, but not in all cases (Harker et al., 1996; Tripathi et al., 1999). The addition of large quantities of acetate with a simultaneous deficiency of nitrogen causes a high C/N ratio, which is favourable for astaxanthin biosynthesis (Orosa et al., 2005; Schoefs et al., 2001). In contrast to the above, Boussiba and Vonshak (1991) expressed the opinion that the concentration of nitrogen in the medium has no influence on astaxanthin formation, indicating, that this process is still insufficiently understood. A deficit of phosphorus in the medium has a comparable effect to lack of nitrogen, but is not so significant.

The concentration of Fe^{2+} only increases astaxanthin production at higher levels (75 μM), but causes growth inhibition (Harker et al., 1996). A low level of iron (18 μM) in the medium has no pronounced influence on growth or carotenoid biosynthesis (Wang et al., 2004).

The results presented here may be helpful in finding additional physiologically active substances that regulate the metabolism of algal cells, causing them to degrade chlorophylls and produce secondary carotenoids. The disappearance of chlorophyll, induced by various sugars, as described in this paper, seems to be an important step related with the biosynthesis of secondary carotenoids. The experiments described here may lead to a better understanding of sugar-induced changes in carotenoid patterns and may be helpful in finding additional factors intensifying astaxanthin biosynthesis.

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PHYSIOLOGICAL REACTION OF LEGUME PLANTS TO INOCULATION WITH ALGAL-RHIZOBIAL ASSOCIATIONS

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The physiological reaction of legume plants to inoculation with algal-rhizobial associations was studied, based on the nodule bacteria, their Tn5 mutants and the cyanobacterium *Nostoc punctiforme*. It was shown that binary inoculation with rhizobia and cyanobacteria may have a positive effect if the inoculation agents and their ratio are correctly chosen. The data obtained on the effect of complex bacterization on the development and productivity of plants under legume-rhizobial symbiotic conditions indicate the prospects of bacterial preparations based on cyanobacteria and rhizobia, including their genetically modified strains.

Key words: legume plants, algal-rhizobial associations, symbiotic efficiency

Introduction

Cyanobacteria are promising objects for biotechnology due to their capacity for photosynthesis, nitrogen fixation and the synthesis of many biologically active compounds, which influence soil biotic conditions (Gromov, 1996). Among the biologically active substances produced by cyanobacteria, bactericidal compounds and antibiotics with herbicidal activity have been discovered (Gromov, 1996). In nature cyanobacteria are closely connected with bacteria, which inhabit their mucilage, for example *Rhizobium, Agrobacterium* and *Pseudomonas*, and are able to form stable associations with them (Pankratova et al., 2008). The application of biological preparations based on nitrogen-fixing microorganisms, particularly nodule bacteria, is one way to increase plant productivity while also conserving soil fertility and protecting the environment (Sytnikov et al., 2007). The creation and selection of compatible algal-rhizobial associations, which include axenic cultures of cyanobacteria and nodule bacteria, may be one method of biologically stimulating the productivity

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of legume-rhizobial symbioses (Pankratova et al., 2008). It might increase the significance of plant-rhizobia interactions and the efficiency of bacterial preparations created on this basis.

Seed germination, germinating vigour and the efficiency of legume symbiotic systems were studied after inoculation with algal-rhizobial associations based on nodule bacteria and their Tn5 mutants with the cyanobacterium *Nostoc punctiforme.*

Materials and methods

Soybean, *Glycine max* (L.) Merr. (cv. Maryana), and lucerne, *Medicago sativa* L. (cv. Yaroslavna), were inoculated with algal-rhizobial associations based on *Bradyrhizobium japonicum* (standard strain 634b, Tn-5 mutants of strain 646 – T66 and T118), *Sinorhizobium meliloti* (standard strains 425a and 441, Tn-5 mutants of strain 425a – T37, T38 and T39) and cyanobacterium *Nostoc punctiforme* (Kütz) Hariot. The Tn5 mutants of nodule bacteria were obtained using the conjugation of *Escherichia coli* with the pSUP2021::Tn5 plasmid and rhizobia with the method described by Novikova et al. (1986). The mutants used in the present research were selected on the basis of their symbiotic activity indices (Vorobey et al., 2007).

Slow-growing soybean nodule bacteria were cultivated on mannite-yeast medium (Child, 1975) at 28°C for 8 days. Fast-growing lucerne nodule bacteria were grown on 79 medium (Allen, 1959) at 28°C for 3 days. Cyanobacteria were cultivated on Fitzgerald medium as modified by Zender and Gorham (1960) in Erlenmeyer test-tubes at 22±2°C and an illumination level of 4500–5000 lux till the stationary growth stage. Algal-rhizobial associations were prepared by mixing suspensions of nodule bacteria or their Tn5 mutants (1×10^8 cell/ml) with cyanobacteria (C_{chl} = 3241.2 ± 16.7 μg/l, $\Delta F = 0.128$) in various ratios (1 : 1, 1 : 2, 2 : 1) directly prior to inoculation. The concentration of chlorophyll (C_{chl}) in the algal cells was detected by the differential fluorometry method using a Planctofluorometer (type: FL300 3M). The potential photosynthetic activity (ΔF) was detected as the difference in fluorescence intensity prior to and after the addition of simasin – the inhibitor of electron transport in photosynthetic cells (Gold et al., 1984).

In order to test germination ability the seeds were grown in sterile Petri dishes (50 seeds per dish) on filter paper wetted with sterile tap water. The seeds were surface sterilized with 70% ethanol for 20 min, washed with sterile tap water for 1 h and inoculated with algal-rhizobial associations based on soybean nodule bacteria, their Tn5 mutants and cyanobacteria. The experiments were performed in four replicates. Germination vigour and seed germination were defined according to the state standard GOST 12038–84 (Loboda et al., 1991).

Lucerne was grown in sterile test-tubes according to the method of Fedorov et al. (1986). The seeds were sterilized for 3 min with concentrated sulphuric acid and washed with sterile tap water for 2 h. They were then grown on agar in Petri dishes at 28°C till the appearance of 3–6 mm roots. The seedlings were cultivated in test-tubes (80 ml) on perlite with Krasilnikov-Korenyako medium and micronutrients, without nitrogen. The inoculation suspensions of cyanobacteria, nodule bacteria and their associations were added (1 ml) to the test-tubes after 24 h. The plants were grown in test-tubes on cotton wool pods at 20–25°C, with a 16-hour photoperiod and illumination at 40000 lux. After 30 days the test-tubes were sealed with special corks prior to the addition of acetylene at a concentration of 10% of total volume. The acetylene reduction activity of the test-tube contents was estimated over 24 h. The experiments were performed in five replicates.

Experiments were also performed under model conditions in the nursery of the Institute of Plant Physiology and Genetics. The plants were grown in 3.5-kg Wagner pots (six plants per pot) in natural daylight. The pots were preliminarily sterilized with a 20% solution of H_2O_2. Washed river sand containing Hellriegel's nutrient mixture was used as a substrate (60% humidity). This mixture contained 25% of the normal nitrogen rate (NNR); 1 NNR corresponded to 708 mg

$Ca(NO_3)_2 \times 4H_2O$ per kg of sand (Grodzinskii and Grodzinskii, 1973). Prior to sowing, the seeds were sterilized with concentrated sulphuric acid for 3 min, washed with running tap water for 2 h, then inoculated with various ratios of algal-rhizobial associations for 1 h. The plant material was sampled at the shooting stage (35 days after seedling emergence).

Nitrogen fixation (nitrogenase activity), estimated as the level of acetylene-reducing activity of the root nodules, was determined by the acetylene-ethylene assay (Hardy et al., 1968) and expressed as nmoles of ethylene formed by the nodules of one plant or in one test-tube for 1 h. The gas mixture was analysed using a Chromatograf-504 gas chromatograph (Mera Elwro, Poland) equipped with a flame-ionization detector. The determinations were performed in five biological replicates.

All results were statistically processed. The data in the tables represent mean values and their standard errors.

Results and discussion

Germination, an important indicator of the quality of seed material, is characterized as the quantity of seeds that sprout during a certain period of time under optimal growing conditions (Loboda et al., 1991). The presence of rhizobia and cyanobacteria in the growth medium may change the optimal growing conditions of the seeds and be reflected in seedling formation. Seed germination (6^{th} day) and germination vigour, i.e. the number of seeds with normal sprouting during a shorter time period (3 days), were determined according to the state standard, GOST 12038–84 (Loboda et al., 1991).

The germination rate and germination vigour of the seeds, and the length and weight of the seedlings increased after the treatment of soybean seeds with *N. punctiforme* alone (Table 1). These indices were not substantially different from the control, inoculated with rhizobia and their mutants. However, the use of algal-rhizobial associations formed from *N. punctiforme* and nodule bacteria (strains 634b, T66 and T118) led in some cases to the enhancement of both seed germination vigour and the length and weight of the seedlings. The germination indices of variants treated with *N. punctiforme* + Tn-5 mutant associations exceeded both the control values and those of the *N. punctiforme* monoculture (Table 1).

Thus, the inoculation of soybean seeds with algal-rhizobial associations based on rhizobia and cyanobacteria promoted seed germination and may have a positive influence on the development of soybean seedlings.

Lucerne plants grown under sterile conditions *in vitro* were inoculated with *N. punctiforme*, standard strain *S. meliloti* 425a and its Tn-5 mutants – T38, T39 and T37 (Table 2). Nitrogen-fixing activity was maximum after *N. punctiforme* inoculation, although the plants in these test-tubes did not form nodules. Nitrogen fixation by *N. punctiforme* exceeded the analogous indices of nodules formed after inoculation with standard strain *S. meliloti* 425a.

It was established that the influence of inoculating plants with algal-rhizobial associations of certain Tn-5 mutants and *N. punctiforme* may differ greatly from the effect of associations based on the standard strain *S. meliloti*

425a. For example, the nitrogen-fixing activity of the Tn-5 mutant T37 + *N. punctiforme* association was higher than that of associations of strain 425a with algae. Thus, combined inoculation with certain rhizobia strains and algae might have a positive effect on the nitrogen fixation activity of legume nodules.

Table 3 shows that in pot experiments the application of algal-rhizobial associations (*N. punctiforme* + standard strains of *S. meliloti*) with different ratios of inoculation agents decreased the level of nitrogen fixation activity in the nodules. In spite of this, binary inoculation with analytically selected strains and algae could have a beneficial effect. Certain associations of *N. punctiforme* with standard rhizobia strains 425a and 441 had a stimulatory effect on nodulation activity, increasing the weight of the aboveground organs and root system compared to the control (Table 3). Binary associations involving a 1 : 1 ratio of *S. meliloti* 425a + *N. punctiforme* and a 2 : 1 ratio of *S. meliloti* 441 + *N. punctiforme* were selected for further investigations.

Table 1
Development of soybean seedlings after seed inoculation with algal-rhizobial associations

Variants	Germinated seeds				Length of seedlings		Weight of seedlings	
	3rd day		6th day					
	No.	%	No.	%	mm	%*	mg	%*
Control (water)	29.5 ± 1.22	59	37.5 ± 1.57	75	47.8 ± 3.8	100	95.6 ± 1.76	100
Nostoc punctiforme	33.0 ± 0.58	66	42.0 ± 0.82	84	57.9 ± 4.1	121	117.2 ± 2.9	123
B. japonicum 634b	31.5 ± 0.74	63	40.0 ± 2.08	80	45.1 ± 3.7	94	106.9 ± 7.7	112
634b+ *N. punctiforme* (1 : 1)	32.5 ± 1.65	65	41.5 ± 2.11	83	56.6 ± 3.0	118	106.8 ± 3.3	112
Tn-5 mutant T66	31.5 ± 2.70	63	37.0 ± 1.35	74	46.8 ± 2.9	98	98.7 ± 3.4	103
T66+ *N. punctiforme* (1 : 1)	33.0 ± 1.63	66	44.0 ± 1.73	88	56.8 ± 4.1	119	106.8 ± 3.3	112
Tn-5 mutant T118	28.5 ± 1.70	57	33.5 ± 2.26	67	43.2 ± 3.01	90	98.7 ± 3.0	103
T118+ *N. punctiforme* (1 : 1)	31.0 ± 1.80	62	42.0 ± 0.58	84	51.3 ± 3.7	107	115.1 ± 5.8	120

* % of control

Table 2
Nitrogen-fixing activity of *Nostoc punctiforme* and lucerne nodules formed by strain *Sinorhizobium meliloti* 425a and its Tn-5 mutants in sterile test-tubes

Variants	nmole of C_2H_4/h per test-tube
Control (without inoculation)	0.00 ± 0.00
Nostoc punctiforme	38.57 ± 2.33
Sinorhizobium meliloti 425a	11.51 ± 0.30
S. meliloti 425a + *N. punctiforme* (1 : 1)	7.61 ± 0.39
Tn-5 mutant T37	13.47 ± 0.87
T37 + *N. punctiforme* (1 : 1)	17.40 ± 1.18
Tn-5 mutant T38	18.43 ± 0.73
T38 + *N. punctiforme* (1 : 1)	8.48 ± 0.49
Tn-5 mutant T39	12.13 ± 0.78
T39 + *N. punctiforme* (1 : 1)	No nodules developed

Table 3

Nitrogen-fixation activity (nmol C_2H_4/h per plant), number of nodules/plant and biomass of lucerne plants after inoculation with algal-rhizobial associations with different ratios of inoculation agents (35 days after seedling emergence)

Variants	Aboveground weight (g)	Root weight (g)	No. of nodules	Nitrogen-fixing activity
Control (water)	0.28 ± 0.02	0.36 ± 0.01	0.00 ± 0.00	–
Nostoc punctiforme	0.55 ± 0.02	0.69 ± 0.02	0.00 ± 0.00	–
Sinorhizobium meliloti 425a	0.60 ± 0.02	0.61 ± 0.02	25.30 ± 1.33	472.42 ± 52.82
425a + *N. punctiforme* (1 : 1)	0.65 ± 0.02	0.65 ± 0.01	31.60 ± 1.60	234.29 ± 14.30
425a + *N. punctiforme* (1 : 2)	0.56 ± 0.02	0.58 ± 0.03	26.40 ± 1.70	85.80 ± 10.07
425a + *N. punctiforme* (2 : 1)	0.55 ± 0.03	0.58 ± 0.03	25.30 ± 1.40	81.18 ± 11.01
Sinorhizobium meliloti 441	0.43 ± 0.02	0.45 ± 0.03	14.30 ± 1.90	400.50 ± 44.62
441 + *N. punctiforme* (1 : 1)	0.47 ± 0.02	0.46 ± 0.02	20.10 ± 1.10	120.35 ± 10.55
441 + *N. punctiforme* (1 : 2)	0.39 ± 0.02	0.39 ± 0.03	13.60 ± 1.90	122.16 ± 8.38
441 + *N. punctiforme* (2 : 1)	0.49 ± 0.03	0.54 ± 0.02	14.60 ± 1.30	297.3 ± 34.10

Thus, a positive interaction between algae and rhizobia is only possible if the inoculation agents and their ratio are optimally selected. The data obtained will be used in future to study the influence of complex bacterization on plant development and productivity under legume-rhizobial symbiotic conditions in order to create bacterial preparations based on cyanobacteria and active rhizobia strains, including genetically modified ones.

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Short communication

Nannochloropsis oculata AS A SOURCE FOR ANIMAL FEED

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Nannochloropsis oculata was evaluated as a source for animal feed. Protein, carbohydrate, mineral and amino acid analyses were performed on samples before and after lipid extraction, and compared with soybean meal and steam-flaked maize. *Nannochloropsis* appears to have an excellent composition for use as a supplement for poultry, cattle or swine. The algae meal contains all the essential amino acids required for animal feed. It also showed comparable amounts of nutrients before and after lipid extraction. The initial review is encouraging, suggesting subsequent animal feeding studies to document digestibility, weight gain and animal acceptance.

Algae have emerged as one of the most promising sources for biodiesel production. Even though no traditional land crop presents a complete solution, microalgae promise to address numerous limiting factors of biofuels. This is increasingly important as the price of maize, soy, rice and other biofuel sources continues to increase. The growth and productivity of algae is significantly greater than that of crops like soybeans, and algae production does not compete for agricultural lands. Algae production facilities are closed and do not require soil for growth, use 99% less water than conventional agriculture, and can be located on non-agricultural land far from water (Chisti, 2007).

Nannochloropsis oculata is a marine unicellular alga commonly cultivated in aquaculture industries for growing rotifers. One of the very important economic issues in algae growth and production is the ability to use algae residue after lipid extraction for animal feed. In this study the nutritional characteristics of algae before and after extraction were compared with those of steam-flaked maize and soybean meal.

Samples of soybean-meal, ground steam-flaked maize, whole *N. oculata*, and the algal residue remaining after fatty acid extraction were sent to two independent commercial laboratories for chemical analysis of fibre, protein, mineral and energy composition. These samples were also sent to a separate laboratory for analysis of amino acid composition.

Table 1

Chemical composition[a] of soybean meal, steam-flaked maize, whole *Nannochloropsis oculata*, and *N. oculata* residue after lipid extraction for biodiesel

Item	Soybean meal	Steam-flaked maize	Whole *N. oculata*	Extracted *N. oculata*
DM, % as fed	92.01	94.91	62.09	70.77
ADF, %	5.89	2.92	2.68	6.64
NDF, %	11.45	9.59	6.95	25.12
Lignin, %	0.66	0.93	0.76	1.81
Non-fibrous carbohydrate	30.58	77.15	60.71	33.86
Extractable fatty acids, %	1.10	3.00	19.70	ND
CP, %	51.55	8.86	28.85	35.28
N fractions, % CP				
Soluble CP (A and B_1)	20.07	32.70	30.38	20.32
B_2	77.51	46.86	14.96	6.49
B_3	1.82	11.92	54.59	63.52
C	0.60	8.52	0.07	9.67
Minerals				
Ash, %	6.95	1.26	8.11	11.51
Ca, %	0.46	0.01	0.23	0.40
P, %	0.78	0.30	0.68	0.73
Mg, %	0.34	0.13	0.38	0.40
K, %	2.59	0.39	1.11	4.51
S, %	0.49	0.12	0.54	0.56
Na, %	0.05	0.01	1.31	0.51
Cl, %	0.05	0.05	1.29	0.26
Mn, ppm	68.80	7.99	24.15	30.45
Zn, ppm	72.95	29.50	201.00	278.00
Cu, ppm	23.10	6.27	21.30	23.95
Fe, ppm	151.50	55.95	217.50	289.50
Mo, ppm	7.86	ND	ND	ND
Co, ppm	ND	ND	ND	ND
Se, ppm	0.52	0.07	0.01	0.02
I, ppm	0.37	0.41	5.30	5.43
Energy				
TDN	80.41	87.91	86.00	79.04
NEM, MJ/kg	9.04	9.01	8.95	7.08
NEG, MJ/kg	6.18	6.17	6.18	4.48
NEL, MJ/kg	7.74	8.60	8.49	7.72

[a]All values are reported on a dry matter basis; ND: Not detectable.

Abbreviations: DM = dry matter; CP = crude protein; TDN = total digestible nutrients; ADF = Acid detergent fibre; NDF = Neutral detergent fibre; NEM = Netto energy maintenance; NEG = Netto energy growth; NEL = Netto energy lactation

Results from the analysis of the feedstuffs were promising. In the United States, soybean meal and maize are the major constituents of most commercial swine and poultry diets. Additionally, maize and soybean meal are also used quite extensively in the dairy and feedlot industries. Soybean meal and maize samples were analysed to serve as quality assurance of the analytical procedures. All the values for maize and soybean meal appear to be similar to the values reported by NRC (1994; 1998; 2000; 2001).

Most nutrient concentrations were increased, as expected, after lipid extraction from *N. oculata* (Table 1), which yields a feedstuff that is intermediate in protein concentrations, with very little alteration in the amino acid profile (Table 2). While previous studies have shown the nutritional value and safety of feeding *Nannochloropsis* sp. to rats (Markovits et al., 1992), there is a paucity of literature regarding its acceptability and potential use for food animals. With the current evaluation it appears that extracted *N. oculata* would serve as an acceptable protein and mineral supplement for livestock diets. Current efforts are being directed to manipulate the mineral content to increase the acceptability of *N. oculata* as a feedstuff and to defray production expenses.

Table 2

Amino acid composition (% of dry matter) of soybean meal, steam-flaked maize, whole *N. oculata,* and *N. oculata* residue after lipid extraction for biodiesel

Item	Soybean meal	Steam-flaked maize	Whole *N. oculata*	Extracted *N. oculata*
L-Arginine	4.11	0.41	1.18	0.79
L-Alanine	2.69	0.78	1.55	1.60
L-Aspartic acid	6.13	0.60	1.66	1.71
L-Glutamic acid	12.66	2.01	2.54	2.73
Glycine	2.55	0.41	1.32	1.32
L-Histidine	1.56	0.33	0.50	0.44
L-Isoleucine	3.00	0.38	0.97	0.96
L-Leucine	4.67	1.20	1.91	1.93
L-Lysine	3.75	0.30	1.34	1.36
L-Methionine	1.22	0.29	0.69	0.72
L-Cysteine	1.21	0.31	0.36	0.24
L-Phenylalanine	3.13	0.44	0.97	1.01
L-Proline	3.10	0.87	0.98	0.96
L-Serine	2.57	0.39	0.83	0.89
L-Threonine	2.26	0.33	1.10	1.10
L-Tyrosine	2.31	0.34	0.68	0.67
L-Valine	3.14	0.50	1.37	1.37
O-Phosphoethanolamine	0.14	0.33	0.28	0.28

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Short communication

FAST AND UNAMBIGUOUS DETERMINATION OF EPA AND DHA CONTENT IN OIL OF SELECTED STRAINS OF ALGAE AND CYANOBACTERIA

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Microalgae may contain large quantities of high-quality EPA and DHA. Therefore, they are considered as a potential source of these important fatty acids. Microalgae can be grown autotrophically on cheap substrates with light. This type of cultivation can be used to maximize the EPA and DHA content in microalgae, making the production of EPA and DHA possible on a large scale. In the present study ten different microalgae were screened for EPA and DHA contents as possible candidates for cultivation in bioreactors.

Key words: microalgae, fatty acids, GC, GC-MS

Introduction

Human physiology depends in various ways on polyunsaturated fatty acids (PUFAs), either as components of membrane phospholipids in specific tissues or as precursors of hormone-like compounds known as eicosanoids (Patil and Gislerød, 2006; Jump, 2002), which have a number of nutraceutical and pharmaceutical applications (Shahidi and Wanasundara, 1998; Horrocks and Yeo, 1999). Eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) are important n-3 PUFAs, while arachidonic acid (AA, C20:4n-6) is a vitally important n-6 PUFA. EPA and DHA show beneficial effects in the courses of diseases like arthrosclerosis, cancer, rheumatic arthritis, psoriasis and diseases of old age such as Alzheimer's and age-related macular degeneration (Devron et al., 1993; Simopoulos et al., 1999).

Fish oils are the major source of PUFAs, and considerable evidence has indicated that the n-3 PUFAs in fish oils are actually derived via the marine food chain from zooplankton that consume n-3 PUFA-synthesizing microalgae (Yongmanitchai and Ward, 1989). Linoleic acid (LA, C18:2n-6) and α-linolenic acid (ALA, C18:3n-3) are predominant in green vegetables and some plant oils.

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Although some research (Carnielli et al., 1996; Salem et al., 1996) has determined qualitatively that humans can convert the parent ALA to EPA and then to DHA, the most recent consensus is the degree of conversion is 'unreliable and restricted' (Gerster, 1998).

As fish oil fails to meet the increasing demand for purified n-3 fatty acids, the demand for alternative sources is increasing. Microalgae may contain large quantities of high-quality EPA and they are considered a potential source of this important fatty acid. Microalgae grow autotrophically on cheap substrates with light. This mode of cultivation can be well controlled and provides a possibility to maximize EPA production on a large scale. Numerous strategies have been investigated for the commercial production of EPA by microalgae. These include screening for high EPA-yielding microalgal strains, strain improvement by genetic manipulation, optimization of culture conditions and development of efficient cultivation systems (Wen and Chen, 2003). In the present study ten different microalgae were screened for their EPA and DHA content. Problems arising during the analysis of the fatty acid profile are also discussed.

Materials and methods

The microalgal strains were cultivated in 2 L glass bubble columns 8 cm in diameter, at a temperature of 25°C, an aeration rate of 2 vvm (mixture of air and 2% carbon dioxide v/v) and a light intensity of 70 $\mu E\ m^{-2}\ s^{-1}$. BG-11 medium was used for the cyanobacterium (Allen, 1959), and diatom f/2 medium for the diatoms, according to Guillard and Ryther (1962). *Porphyridium* was cultivated in the medium reported by Sommerfield and Nichols (1970). The AF6 medium was used for *Chlorella sorokiniana, Scenedesmus pectinatus, Monodus subterraneus* and *Neochloris oleoabundans* according to Kato (1982), while *Chlorella minutissima* and *Nannochloropsis* sp. were cultivated using an enriched natural seawater medium (Provasoli, 1968).

The procedure for sample preparation consisted of lipid extraction at room temperature with chloroform, methanol and water according to the method of Bligh and Dyer (1959). Ten different strains of microalgae were lyophilized and 40 mg of the freeze-dried powders were used for lipid extraction with a mixture of 7.6 mL MeOH/$CHCl_3$/H_2O (2:1:0.8 v:v:v). After final removal of the solvents in a gentle stream of nitrogen, a green, crude extract was obtained. The derivatization was done by the introduction of 150 µL toluene and 100 µL trimethyl-sulphonium hydroxide (TMSH) reagent. The vial was gently hand-shaken until a homogeneous solution was obtained.

The analysis of fatty acids with gas chromatography (GC) was carried out using a Hewlett-Packard HP 5890 Series II gas chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a split/splitless injector and an Agilent 7673 series auto-sampler. Detection involved a combination of flame ionization detection (FID) and mass spectrometry (MS) (MS Engine HP-5989B, Agilent Technologies, Waldbronn, Germany). The separation of fatty acid methyl esters was achieved on a SP2380 fused silica column (Supelco, Bellefonte, PA, USA) 60 m × 0.32 mm i.d., 0.2 µm film thickness. The injection volume was 2 µL at a split ratio of 1:30.

Results

As a consequence of overloading the capillary column, the retention times of the respective signals for EPA in the sample chromatograms (GC-FID) shifted compared to the retention times of a standard solution containing several fatty

acids. Therefore, the algorithm for automatic integration included in the software package was no longer able to denote these high area peaks as EPA. Thus, the chromatograms of *Phaeodactylum tricornutum*, *Nannochloropsis* sp. and *Chlorella minutissima* suggested the absence of EPA and the presence of C24:0 instead (Table 1). In order to remedy these deficiencies, all samples were reanalyzed by GC-MS in scan mode (Fig. 1), when the presence or absence of each fatty acid could be unambiguously determined. The results obtained with GC-MS were used to modify and refine the algorithm for the automatic integration of GC-FID. The exact quantitation of EPA and DHA was then possible from the signals obtained with GC-FID (Table 2).

Table 1
Percentage of EPA in selected microalgae before and after consideration of GC-MS data

Algae	Results (%)[a]	
	EPA[b]	EPA[c]
Thalassiosira punctigera	18.919	18.948
Neochloris oleoabundans	0.824	0.521
Phaeodactylum tricornutum	not detected	19.818
Nannochloropsis sp.	not detected	21.096
Nostoc sp.	not detected	not detected
Chlorella minutissima	not detected	24.910
Chlorella sorokiniana	0.125	0.171
Monodus subterraneus	31.567	20.169
Scenedesmus pectinatus	0.962	0.411
Porphyridium cruentum	16.188	15.883

[a]: percentage of total fatty acids; [b]: results obtained from GC-FID without consideration of GC-MS data; [c]: results obtained from GC-FID with consideration of GC-MS data

Table 2
EPA and DHA content in selected microalgae μg/g dry weight

Algae	Content (μg/g)	
	EPA	DHA
Thalassiosira punctigera	3441.8	675.2
Neochloris oleoabundans	189.5	169.8
Phaeodactylum tricornutum	12173.6	1121.0
Nannochloropsis sp.	18170.4	118.1
Nostoc sp.	not detected	114.2
Chlorella minutissima	30088.5	111.7
Chlorella sorokiniana	30.6	87.1
Monodus subterraneus	3170.4	99.1
Scenedesmus pectinatus	159.2	115.8
Porphyridium cruentum	2930.6	91.8

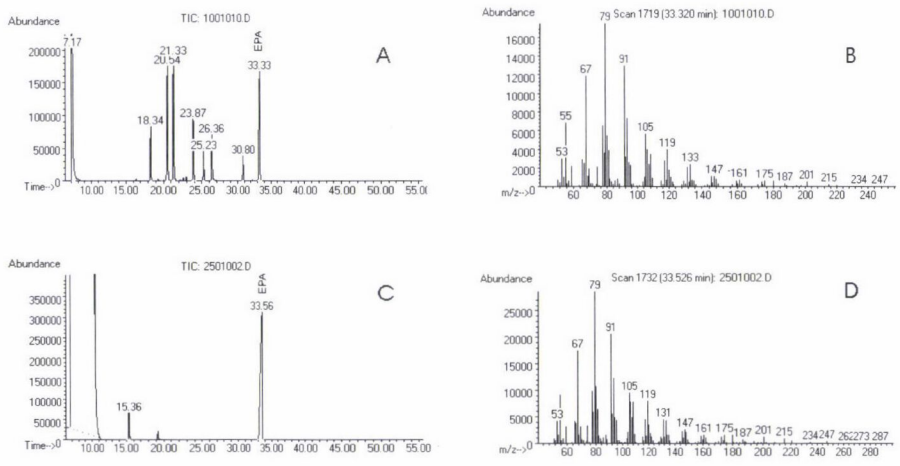

Fig. 1 (A) GC-MS of *Chlorella minutissima* – TIC; (B) mass spectrum of peaks at a retention time of 33.3 min in chromatogram A; (C) GC-MS of an EPA standard solution (1766 µmol/L in toluene) – TIC; (D) mass spectrum of peaks at a retention time of 33.5 min in chromatogram C

Discussion

It was shown that the retention times in fatty acid profiles may shift depending on the amounts injected. Especially when large amounts are injected, an overload may result, with higher retention times of the peaks. The algorithms for automatic integration implemented in the software packages are then no longer capable of denoting the corresponding signals correctly. In such cases, the presence of each fatty acid should be confirmed by mass spectrometry and the calibration files containing the retention times of each signal should then be modified.

The screening of ten different microalgae for EPA and DHA contents revealed three candidates with high amounts of these poly-unsaturated fatty acids (PUFAs): *Phaeodactylum tricornutum*, *Nannochloropsis* sp. and *Chlorella minutissima.* These three species of microalgae had contents between 12 and 30 mg/g for EPA and 112 to 1121 µg/g for DHA (Table 2). The EPA contents obtained for the other seven species investigated were substantially lower (< 3.5 mg/g dry weight), while the DHA content in the other species ranged from 87 µg/g (*Chlorella sorokiniana*) to 675 µg/g (*Thalassiosira punctigera*). Therefore, *Phaeodactylum tricornutum*, *Nannochloropsis* sp. and *Chlorella minutissima* are promising candidates for the exploitation of "PUFA-rich" oils through the cultivation of microalgae in bioreactors. *Thalassiosira punctigera* would be another option if oils with a high percentage of DHA were favoured.

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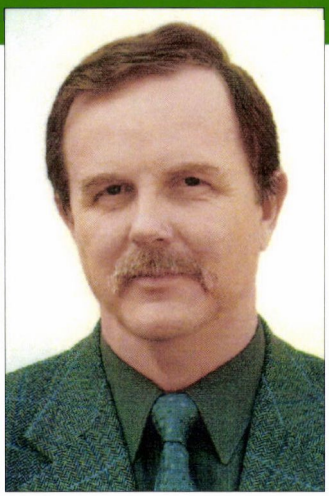

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PERFORMANCE EVALUATION AND GENETIC ANALYSIS OF MAIZE POPULATIONS AND DIALLEL CROSSES UNDER IRRIGATED AND DROUGHT-STRESSED CONDITIONS

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This study aimed to assess the response of eight maize populations and their 28 diallel crosses to water stress and conventional irrigation. The source populations consisted of maize germplasm from CIMMYT and the F_2 generation of commercial single-cross hybrids. The trials were conducted at three locations in two successive years. Six characteristics relevant to drought stress were recorded. On average, water stress reduced the plant height and ear attachment height, and increased the number of days to silking and the anthesis to silking interval (ASI). The reductions were greater in the diallel crosses, and the increases in the parental populations. At one location, where irrigated and drought-stressed treatments were established side by side, diallel analysis for yield revealed that: (i) the control (B73 × Mo17) and the commercial hybrid *Costanza* had significantly reduced yield under drought stress; (ii) the general (GCA) and specific (SCA) combining ability were significantly higher in the irrigated experiments; and (iii) populations derived from the F_2 generation showed higher GCA.

Keywords: ASI (anthesis to silking interval), GCA (general combining ability), SCA (specific combining ability), plant height, ear attachment height

Introduction

Drought is the second most significant cause of yield loss in farmers' fields after low fertility (Edmeades et al., 1994). Short-duration drought is an abiotic constraint contributing to fluctuations in maize (*Zea mays* L.) yields in any environment. Periodic droughts may occur at the most drought-sensitive developmental stages of the crop, such as flowering and grain filling (Nesmith and Ritchie, 1992). Robins and Domingo (1953) reported a yield reduction of up to 22%, even when drought stress lasted for just one or two days at the time of tasselling or pollination. Several traits such as leaf rolling, best-adapted root system, increased pubescence, etc. contribute to dehydration avoidance and

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tolerance, which have also been found to be positively associated with yield under stress (Acevedo and Ceccarelli, 1989). For maize, the criteria used for selection under drought were: delayed foliar senescence, high osmotic potential, high grain yield and ears per plant under stress, short anthesis to silking interval (ASI), and lodging resistance under stress (Edmeades et al., 1994).

The high degree of heterogeneity in the intensity and timing of drought stress may render selection ineffective and limit the progress from selection (Byrne et al., 1995). One way to develop drought-tolerant maize genotypes is to select under conditions of moisture stress (Johnson and Geadelmann, 1989). However, several studies have indicated that progress from recurrent mass selection for grain yield in maize may be affected by stress conditions in the environments during selection (Hallauer and Miranda, 1981). Hallauer and Sears (1969) suggested that competition effects due to moisture stress during mass selection might have been responsible for the lack of progress observed in two open-pollinated varieties. Rosielle and Hamblin (1981) presented theoretical arguments that selection for tolerance of stress, when compared to direct selection for productivity, will generally result in reduced productivity in non-stressed environments and lower mean productivity in both stressed and non-stressed environments. Gardner and Stevens (1988) reported that when families were tested under both irrigated and non-irrigated conditions, major emphasis was placed on performance under favourable conditions.

The utilization of morphological characteristics is very useful in selection for resistance under decreased quantities of water. An extensive programme carried out by CIMMYT (Edmeades et al., 1994) went through eight cycles of recurrent full-sib selection for drought tolerance, and resulted in a yield increase of 500–800 kg ha^{-1}. This selection strategy proved to be effective for improving yield under drought stress and for developing high-yielding maize germplasm with stable performance across wide environmental conditions (Byrne et al., 1995; Banziger et al., 1999). Modern elite germplasm performs better than less improved, older counterparts, and much of the observed genetic gain in yield during the past 30 years has been attributed to greater stress tolerance rather than to an increase in yield potential *per se* (Duvick, 1992; Tollenaar and Lee, 2002). Genetic solutions are unlikely to close more than 30% of the gap between potential and realized yield under water stress (Edmeades et al., 2003).

The main objective of this study was to assess the genetic performance of open-pollinated populations, derived from either CIMMYT maize germplasm or the F_2 generation of commercial hybrids and their diallel crosses, as compared with commercial hybrids (used as controls), under irrigated and water-deficit conditions. An additional task was to identify characteristics less affected by the environment, to be used as selection criteria under drought stress.

Materials and methods

Eight maize populations were used as parents in a diallel cross. Four of the populations (V_1, V_6, V_7 and V_8) originated from CIMMYT (1998) germplasm (subtropical and temperate populations developed from a mixture of materials of very different origin, proving their broad genetic base), while the other four (V_2, V_3, V_4 and V_5) were derived from the F_2 generation of commercial single-cross hybrids (Table 2, first row). Three of these were typical dents, widely cultivated by local farmers in the 90s, and possessed all the characteristics of hybrids in the US Corn Belt. The fourth single cross hybrid, V_4 (IS-027) was an old hybrid with flint-type grains. All the populations had been improved for several cycles using the ear-to-row selection methodology for adaptation to local conditions, maturity and resistance (Sfakianakis et al., 1996; Evgenidis et al., 2001). The diallel crosses between the populations were developed in the growing season before the evaluation and repeated in the next year in order to have sufficient seed. Up to two hundred plants from each population were top-crossed with pollen from an equal number of plants from each of the other populations. The eight populations, their 28 diallel crosses and two controls were evaluated for two successive years, i.e. a total of eight randomised complete block designs, with two replications. The commercial single cross hybrid Costanza (PR3245, distributed by Pioneer Hi Bred Hellas), grown on 21% of the total area cultivated with maize in Greece, and the single cross hybrid B73 × Mo17 were used as controls. The experiments were established at three locations: Thessaloniki, Loc. A (40°37' N, 23°00' E, silt-clay soil), Xanthi, Loc. B (41°10' N, 25°08' E, sandy-clay soil) and Ludias, Loc. C (40°42' N, 22°28' E, clay-loam soil). Each plot consisted of two 5 m rows, with row and plant distances of 0.80 m and 0.20 m, respectively, thus giving a final density of 62,500 plants ha^{-1}. All practices, apart from irrigation, were aimed at maximizing yield. At Loc. A two treatments, i.e. irrigated and drought-stressed, were established side by side each year. A drip irrigation system with the drippers in each plant row was used. The irrigation needs were calculated by referring to an evapotranspiration pan, which was placed in the field. The quantities of water applied were estimated with hydrometers. At Loc. B, only a drought-stressed treatment, at roughly 50% of plant needs, was used. At this location the trials were irrigated three times instead of six, at 15–18 days interval, starting on 1st July. At Loc. C only a fully irrigated (by sprinkler) trial was conducted. However, it seems that at Loc. B, the water stress was less than expected in the second year, while at Loc. C parasitic pests influenced the trial in the second year of experimentation and this negatively affected the output.

In addition to yield, which was expressed in t ha^{-1} adjusted to 15.5% grain moisture, the following traits were recorded: (i) plant and ear attachment height (cm), (ii) period from seeding to silking (days), (iii) anthesis to silking interval (ASI, days), (iv) grain moisture at harvest (%) and (v) root lodging (% of total plants). Combined analysis of variance was performed, considering the eight trials as different water stress environments, although the same locations were used. Correlations between the yields in irrigated and water-stressed plots within locations and years were calculated using JMP IN software (Sall and Lehman, 1996).

The results of the two-year experiment at Loc. A were used to estimate the components of genetic parameters from the diallel crosses, and to determine whether these components differed in the drought-stressed environment. Analysis of variance was performed separately for the irrigated and drought-stressed trials. Total yield variance (excluding the controls) was partitioned to the eight populations and their 28 diallel crosses, and the components of the genetic parameters were calculated according to Analysis III, fixed model, of Gardner and Eberhart (1966). The expected variety means (V_i) and variety cross means (C_{ij}) were:

$V_i = \mu_v + v_i$ and $C_{ij} = \mu_c + g_i + g_j + s_{ij}$

where: μ_v = mean of all parents; v_i = contribution of i^{th} parent; μ_c = mean of the crosses; g_i = general combining ability of i^{th} parent; and s_{ij} = specific combining ability of i^{th} and j^{th} parents.

The mean heterosis of all parents is $\bar{h} = \mu_c - \mu_v$, and the mean heterosis of the i^{th} parent $h_i = g_i - 1/2v_i$.

Results

The mean grain yield in the eight environments ranged from 3.26 to 10.16 t ha^{-1} (Table 1). The other traits measured reflected a broad range of environmental conditions. At Loc. B in the second year, the grain yield, plant height and ear attachment height were higher than in the previous year, reflecting the ambiguous effect of moisture stress. On the other hand, at Loc. C in the same year, the results showed very low values, reflecting unfavourable environmental conditions. The combined analysis of variance across the eight environments showed significant effects ($P<0.01$) for the environments and the treatments, as well as for the interaction between them. Bartlett's test of homogeneity between environments for all the traits showed an inconstant variance (data not presented). Despite the significant differences between the environments for all the traits, the rank correlation coefficients for genotypes between the irrigated and drought-stressed trials were significant for all traits, ranging from 0.43* for ASI to 0.91** for silking (data not shown).

Figure 1 depicts the density ellipses of the normal distribution for grain yield in the drought-stressed (Y) and irrigated (X) treatments. The eight environments formed four groups, as follows: (i) Loc. A, 1st year; (ii) Loc. A, 2nd year; (iii) Loc. B + Loc. C, 1st year and (iv) Loc. B + Loc. C, 2nd year. Ignoring the groups the linear correlation was low for all the environments ($r=0.0155$), but when the groups were considered the elliptical contours of normal ($P=0.5$) distribution were differentiated. The three ellipses towards the top of the plot were diagonally oriented. The diagonal flattening of the elliptical contours is an indication of a strong correlation. The correlation was high for environmental groups i, iii and iv (0.70, 0.50 and 0.66, respectively), and very low for environmental group ii (–0.07). On the other hand, the two ellipses at the top of the plot showed a low stress effect in the drought-stressed treatments for environmental groups iii and iv, while at Loc. A in both years (groups i and ii) the genotypes were highly drought-stressed. For this reason the grain yield of Loc. A only was chosen for the estimation of genetic parameters.

Table 1

Mean values of grain yield and seven characteristics of 38 genotypes evaluated in eight environments (locations and years)

Characteristics	First year				Second year				SD
	Loc. A		Loc. C* and B		Loc. A		Loc. C* and B		
	Irrig.	Stress.	Irrig.	Stress.	Irrig.	Stress.	Irrig.	Stress	
Yield (t ha^{-1})	8.20	4.16	10.16	5.01	7.51	3.26	6.27	6.91	0.39
Plant height (cm)	225.1	189.1	–	203.0	199.4	156.8	–	259.7	3.19
Ear height (cm)	108.8	91.9	–	91.9	114.1	93.9	–	127.8	1.85
Anthesis (days)	68.34	69.17	65.22	69.74	69.09	71.0	65.72	73.15	0.28
Silking (days)	71.36	72.73	68.09	73.63	73.23	76.62	69.04	78.05	0.32
ASI (days)	3.03	4.05	2.88	4.16	4.14	5.62	3.88	4.91	0.11
Lodging (ratio)	0.31	0.32	0.14	0.45	0.04	0.06	0.02	0.00	0.02
Grain moisture (%)	21.24	21.24	20.60	18.90	18.18	17.50	21.07	23.57	0.31

*No data for plant and ear attachment height; ASI: Anthesis to silking interval

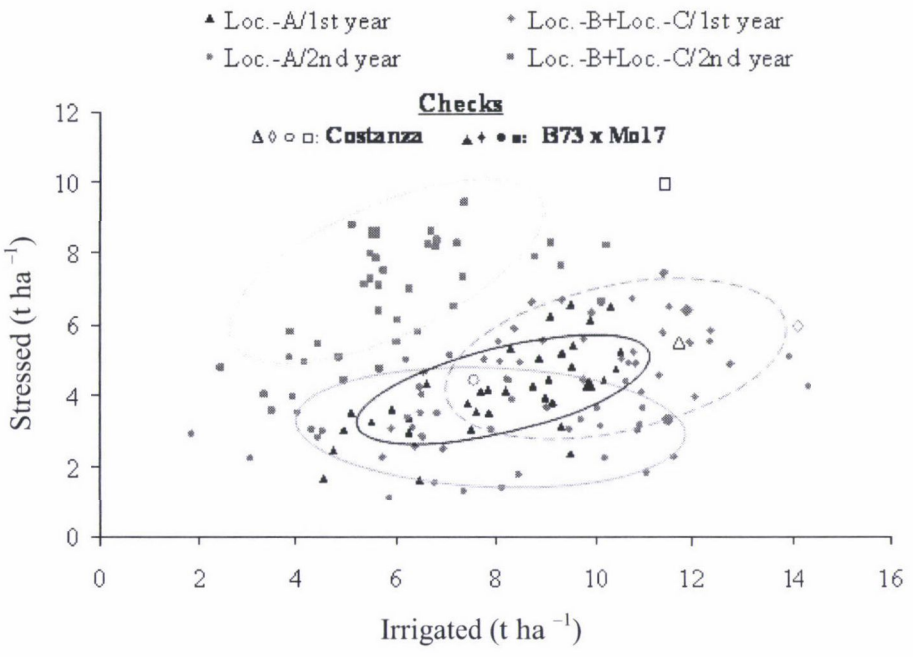

Fig. 1. Density ellipses of normal distribution for the grain yield variable in drought-stressed (Y) and irrigated (X) treatments, grouped into four environments, for the 38 genotypes tested

The mean grain yields of the populations (on the diagonal) and their diallel crosses (above the diagonal) in the irrigated and drought-stressed plots at Loc. A are presented in Table 2. The mean yield of some diallel crosses and the corresponding value of the better performing control hybrid did not differ significantly. Under drought-stressed conditions the highest yielding control (data not shown) was the hybrid B73 × Mo17 (4.96 t ha^{-1}), and under normally irrigated conditions the commercial hybrid Costanza (10.70 t ha^{-1}). The mean yield in the irrigated plots was 7.53 t ha^{-1} for the populations (means of eight diagonal values in the table) and 7.79 t ha^{-1} (means of 28 values above the diagonal in the table) for the diallel crosses, while under drought-stressed conditions it was 2.82 t ha^{-1} for the populations and 3.92 t ha^{-1} for the diallel crosses.

The results of analysis of variance for yield according to this method are presented in Table 3. The mean squares of all the entries, as well as those of the genotypes, varieties (V_i), crosses (C_{ii}), GCA (g_i) and SCA (s_{ii}), were only highly significant ($P<0.01$) for the irrigated plots. The mean squares of the interaction between genotypes and years were significant for both irrigated and drought-stressed plots. By contrast, the mean squares of the genotype by year interaction were not significant in either case. Under drought-stressed conditions the mean squares of the interactions C_{ij} by year, g_i by year and s_{ij} by year were highly

significant or significant. An adequate number of crosses between the populations s_{12}, s_{14}, s_{15}, s_{18}, s_{23}, s_{24} and s_{25} gave similar grain yield to the controls in the normally irrigated environments. The population IS-027 (V_4) and the diallel cross PR-3183 × Capinapolis 8645 RE (s_{28}) exhibited the smallest difference between irrigated and stressed conditions. The population Chuguisaga 8233 showed the highest GCA (g_1, Table 4), while the highest SCA was obtained for the crosses PR-3183 × PX-95 (s_{25}) and Tlaltizapan 8633 × La Platina 8946 (s_{67}) in an irrigated environment. The higher GCA of populations derived from single cross hybrids explains the preference of maize breeders for selection from elite F_2 populations (Jenkins, 1978). Also, data from a study by Fountain and Hallauer (1996) suggest that average genetic variability within F2 populations exceeds that within narrow-base synthetic populations and is equivalent to that within broad-base synthetic populations.

The estimates of genetic constants, according to model III of Gardner and Eberhart (1966) and based on the data in Table 2, are presented in Table 4. The genetic constants of stressed plots are presented for comparison with the corresponding irrigated treatments, since the variety effect, V_i, and the GCA

Table 2

Mean grain yield (t ha^{-1}) of the eight populations and their 28 diallel crosses (on the diagonal and above the diagonal, respectively) for irrigated and drought stressed plots at Loc. A

Variety	V_1 CIMMYT 8633 (Tlaltizapan 8633)	V_2 Hybrid PR3183 (Pioneer Hi-Bred)	V_3 Hybrid B73 × Mo17	V_4 Hybrid IS-027 (FS68 × NE2)	V_5 Hybrid PX-95 (Northrup King Co)	V_6 CIMMYT Pop. 33 (Amarillo subtropical)	V_7 CIMMYT Pop. 46 (Templado Amarillo Cristalino)	V_8 CIMMYT Pop. 45 (Amarillo Bajio)
Normally irrigated								
V_1	7.55	10.24	8.63	10.27	10.11	8.28	9.56	10.19
V_2		6.52	10.74	10.30	10.98	5.99	9.17	7.10
V_3			8.67	4.45	3.91	7.10	7.76	8.11
V_4				5.83	6.84	4.90	4.17	7.54
V_5					9.38	7.02	5.97	5.14
V_6						8.45	8.79	7.37
V_7							6.16	7.34
V_8								7.68
Drought-stressed								
V_1	3.04	4.30	4.13	5.25	4.08	4.87	4.93	3.32
V_2		2.45	4.18	4.19	4.37	3.38	5.12	4.09
V_3			3.27	2.35	2.34	4.15	3.97	4.45
V_4				3.16	3.57	4.33	2.29	5.02
V_5					3.88	3.89	2.93	3.22
V_6						2.84	3.76	3.48
V_7							2.16	3.84
V_8								1.74

Table 3
Analysis of variance on grain yield of eight maize populations and their 28 diallel crosses tested under irrigated and drought-stressed conditions, at Loc. A in two successive years

Source of variation	df	Irrigated		Drought-stressed	
		Mean square	% of total variation	Mean square	% of total variation
Years	1	18.31*	2.13	31.16**	10.09
Replications over years	2	0.37	0.09	1.09	0.71
Entries	37	15.53**	(66.84)	3.24	(38.81)
Controls	1	2.10	0.24	2.65	0.86
Genotypes vs. Control	1	46.04**	5.36	3.80	1.23
Genotypes	35	15.04**	(61.24)	3.24	(36.72)
Varieties (V_i)	7	12.81**	10.44	1.84	4.18
Varieties × Crosses (=ħ)	1	17.64**	2.05	21.48**	6.96
Crosses (C_{ij})	27	15.52**	(48.75)	2.93	(25.59)
GCA (g_i)	7	26.50**	21.58	4.14	9.39
SCA (s_{ij})	20	11.68**	27.17	2.50	16.20
Entries × Years	37	3.92**	(16.88)	2.23**	(26.78)
Contol × Years	1	67.54**	7.86	2.22	0.72
Genotypes × Control × Years	1	2.44	0.28	2.18	0.71
Genotypes × Years	35	2.14	(8.73)	2.24	(25.35)
Varieties × Years	7	3.68*	3.00	0.50	1.13
Varieties × Crosses × Years	1	6.62*	0.77	24.04**	7.79
Crosses × Years	27	1.58	(4.97)	1.88**	(16.44)
GCA ×Years	7	2.11	1.72	3.32*	7.53
SCA × Years	20	1.39	3.24	1.38*	8.91
Error	74	1.63	14.06	1.04	23.62
Total	147		100.00		100.00

** and *; significant differences at p=0.01 and p=0.5, respectively

effect, g_i, were negligible in the drought-stressed treatments (Table 3). The total variety effect for the populations arising from the hybrids $v_{(Hybrid)}$ was positive, in contrast with the total GCA effect for the same populations, $g_{(Hybrid)}$, which was negative. This was also valid for the stressed trials, where the differences were not significant. This means that the populations from hybrids had better performance, but at the same time they were worse as parents. Since the specific heterosis s_{ij} was significant, the higher-yielding diallel crosses s_{25}, s_{67}, s_{23}, s_{24}, s_{14} and s_{38} were selected. However, neither the variety means, v_i, and the general combining ability, g_i, nor the drought-stressed results, should be ignored. Varieties v_5, v_3, and v_6 gave the highest yields in the irrigated treatment, while varieties v_1 and v_2 were selected for their general combining ability.

Discussion

The study was set up on fields in three locations over two years. At the first location, irrigated and drought-stressed treatments were established side by side, giving a total of four environments, while of the other two locations one

was irrigated and the other drought-stressed, giving another four environments. Since the genotype by environment (GE) interaction was higher than expected, causing problems for the main task of the study (to assess the genetic performance of open-pollinated populations and their diallel crosses), it was decided to exclude Loc. B and Loc. C from the diallel study on yield. This decision was based on the fact that in the present experiment the grain yield of all the genotypes evaluated at Loc. A and Loc. C was low compared to that reported by previous researchers (Sfakianakis et al., 1996; Evgenidis et al., 2001). This was attributed to adverse climatic conditions and to a severe attack of rust diseases (*Puccinia* spp.). Thus, a qualitative interaction was observed (Baker, 1988). All these data showed that the evaluation of unstressed and drought-stressed environments was best conducted on experiments carried out in neighbouring fields, as the comparison of stresses occurring in separate locations leads to great uncertainty, particularly in maize. Other authors also reported that the year effect and the variety by year interaction were larger than the location effect and location by year interaction (Simmonds, 1979).

Table 4
Genetic constants estimated from data of Table 2 (Analysis III)

Genotype	Irrigated	Stressed
μv	7.53	2.82
v_1 CIMMYT 8633	0.02	0.22
v_2 Hybrid PR3183	–1.01	–0.36
v_3 Hybrid B73 × Mo17	1.15	0.45
v_4 Hybrid IS-027	–1.70	0.34
v_5 Hybrid PX-95	1.85	1.06
v_6 CIMMYT Pop. 33	0.92	0.02
v_7 CIMMYT Pop. 46	–1.37	–0.66
v_8 CIMMYT Pop. 45	0.15	–1.08
Sum of $V_{(Hybrid)}$	0.28	1.49
Sum of $V_{(CIMMYT)}$	–0.28	–1.49
μc	7.79	3.92
g_1 CIMMYT 8633	2.13	0.57
g_2 Hybrid PR3183	1.67	0.36
g_3 Hybrid B73 × Mo17	–0.63	–0.31
g_4 Hybrid IS-027	–1.01	–0.07
g_5 Hybrid PX-95	–0.75	–0.51
g_6 CIMMYT Pop. 33	–0.84	0.07
g_7 CIMMYT Pop. 46	–0.29	–0.10
g_8 CIMMYT Pop. 45	–0.28	–0.00
Sum of $G_{(Hybrid)}$	–0.72	–0.53
Sum of $G_{(CIMMYT)}$	0.72	0.53

Genotype	Irrigated	Stressed
s_{12}	–1.35	–0.56
s_{13}	–0.65	–0.05
s_{14}	1.36	0.83
s_{15}	0.95	0.10
s_{16}	–0.79	0.31
s_{17}	–0.07	0.54
s_{18}	0.56	–1.17
s_{23}	1.92	0.21
s_{24}	1.84	–0.02
s_{25}	2.28	0.59
s_{26}	–2.62	–0.98
s_{27}	0.00	0.94
s_{28}	–2.07	–0.19
s_{34}	–1.70	–1.19
s_{35}	–2.49	–0.76
s_{36}	0.78	0.48
s_{37}	0.89	0.46
s_{38}	1.24	0.85
s_{45}	0.82	0.23
s_{46}	–1.04	0.41
s_{47}	–2.32	–1.45
s_{48}	1.04	1.18
s_{56}	0.83	0.41
s_{57}	–0.77	–0.38
s_{58}	–1.61	–0.18
s_{67}	2.13	–0.13
s_{68}	0.71	–0.50
s_{78}	0.13	0.02

The analysis of variance for grain yield at Loc. A showed that the total percentage variance was twice as high for irrigated entries than for drought-stressed plots. The significant differences between genotypes vs. controls in the irrigated trials showed that the controls only performed better under irrigated conditions, while the significant differences between populations and diallel crosses in both cases showed the stability of the populations and their crosses under drought stress. None of the genotypes that underwent water stress exceeded the corresponding genotype that was regularly irrigated. From the eight populations, V_5 was the highest yielding under both drought- stressed and irrigated conditions. This population (Hybrid PX-95) performed similarly under irrigated conditions in a previous experiment (Sfakianakis et al., 1996). This could be an indication that non-stressed environments increase the precision and reliability of selection (Duvick, 2005). The best way to affect future gains in yielding ability may be to make further improvements in tolerance of high plant densities, in combination with improvements in potential yield per plant under low stress environments (Duvick, 1997). Testing for drought stress and non-stress in different environments (locations and years) may detect high yield potential, although it is difficult to separate it from stress tolerance and then select for high yield (Evans and Fisher, 1999).

Some of the traits (measured in all environments) were used for the final evaluation of the genotypes. For example, all genotypes in all environments revealed reduced plant and ear attachment height, an increased number of days to anthesis and silking, and an increase in ASI, as the drought stress increased. It seems that delayed flowering is a physiological reaction of stressed plants, but does not mean that it is shifting towards late maturity. On the other hand, the breeder cannot overcome the anthesis to silking interval, which is strongly correlated with grain yield. Bolanos and Edmeades (1993) estimated that an increase in ASI from –0.4 days (where the female inflorescence precedes the male) to 10 days leads to a decrease in grain yield of 8.7% per day. Edmeades et al. (1994) confirmed that ASI is connected with resistance to drought. Lodging and grain moisture were only slightly influenced by irrigation, while they were influenced intensely by the environment. Lodging tended to increase under drought-stressed conditions, and the same was true of grain moisture.

Conclusions

The estimated population/hybrid × environment interaction can be considered to be qualitative, i.e. the interactions complicate selection and the identification of superior genotypes. The significance of GCA and SCA in the irrigated experiments showed that unstressed conditions permit greater differentiation between genotypes. The higher GCA of populations derived from single cross hybrids indicates the additive effect of parental inbred lines. Under Mediterranean conditions a reduction in maize yield is associated more with water input deficits than with biotic stress.

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STUDIES ON THE EFFECT OF N FERTILISATION ON THE GROWTH OF MAIZE (*Zea mays* L.) HYBRIDS II. PLANT GROWTH ANALYSIS AND GROWTH PARAMETERS

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The use of growth analysis and growth parameters could make an important contribution to improving the utilisation of N fertiliser by maize hybrids. In 2001 and 2002 the effect of four N fertiliser rates (0, 80, 160, 240 kg ha^{-1}) on the growth and productivity of three maize hybrids with different vegetation periods was studied in a long-term experiment involving continuous maize, representing a stress environment, set up in Martonvásár on chernozem soil with forest residues almost 50 years ago. Each year eight samples were taken at 14-day intervals for the destructive method of growth analysis. N fertiliser was found to have a significant effect on the growth parameters of individual plants (RGR, NAR, LAR) in both the vegetative and generative stages of growth, up to N rates of 80 and 160 kg ha^{-1}, respectively. The value of RGR increased until the N_{160} treatment and that of NAR until N_{80}, while LAR declined significantly in response to N fertilisation. RLGR was enhanced by N fertiliser up to a rate of N_{80}, and all the N treatments reduced the extent of leaf withering. Among the growth parameters of the canopy, the values of CGR and HI rose significantly up to N_{160} and that of LAI_{max} up to N_{80}. The cumulative values of LAD and BMD were highest in the N_{160} treatment. All the growth parameters increased as the vegetation period of the hybrids lengthened, and all reflected the year effect. Compared to the highest N rate, N stress of 29–38% was calculated for the control treatment. On average, N fertilisation resulted in a 6–27% relative decline in LAI after flowering.

Key words: maize, nitrogen stress, long-term experiment, plant growth analysis, growth parameters

Abbreviations: BMD, biomass duration; CGR, crop growth rate; HI, harvest index; LAD, leaf area duration; LAI, leaf area index; LAR, leaf area ratio; NAR, net assimilation rate; RGR, relative growth rate; RLGR, relative leaf area growth rate; S_N, nitrogen stress.

Introduction

Traditionally the improvement in maize grain yield has been attributed to both genetic gains made by plant breeders and to the adoption of improved agronomic practices. However, it is more realistic to state that the increase in

grain yield is due to the interaction between genetics and agronomic practices (Tollenaar and Lee, 2002). Besides tolerance of abiotic and biotic stresses, the probable cause of high yield in modern hybrids is the larger number of ear-bearing plants per unit land area without a reduction in the kernels per ear. Modern maize hybrids have greater yield potential compared with older hybrids. Tollenaar et al. (1993) showed that the higher yields obtained with modern hybrids can be attributed to higher kernel number per plant and higher aboveground plant growth rates during the period from a week before silking to three weeks after silking.

The identification of the physiological, biochemical or morphological characteristics responsible for inherent or environmentally induced variations in plant growth or yield requires careful growth analysis (Lambers et al., 1989). An analysis of the relative growth rate (RGR), net assimilation rate (NAR) and leaf area ratio (LAR) may lead to a better understanding of variations in growth rate. In the study of McCullough et al. (1994) the N stress index indicated a proportionally greater depression in absolute and specific growth rate for an old maize hybrid compared with a new one. The use of growth analysis and growth parameters has recently been reported in maize physiology research and in yield simulation models (Westgate and Boote, 2000; Ritchie and Alagarswamy, 2003; Westgate et al., 2004). Otegui and Andrade (2000) investigated the relationship between light interception, ear growth and kernel set in maize, and demonstrated that the crop growth rate (CGR) or plant growth rate during the period including flowering gave the best indication of the mechanisms of kernel set and ear plasticity and allowed threshold values to be defined for ear abortion, prolificacy, and limitations on ear morphogenesis. Nitrogen stress, as a quantitative estimate of the intensity of current nitrogen deficiency in a plant, can be evaluated as the proportion by which the growth rate of the plant falls short of the maximum growth rate attained with a non-limiting supply of nitrogen (Greenwood, 1976).

The present investigations indicate that growth analysis is a particularly suitable method for the comparative analysis of the growth of maize plants in terms of dry matter production and of the ecological and agronomic factors influencing growth (Berzsenyi and Dang, 2005; 2007). The aim of the research was to characterise the effect of N fertilisation on the growth of maize hybrids (1) using mean values of growth parameters for the canopy, the whole plant and individual plant organs (stalk, leaf area, grain yield) and (2) using calculated values of N stress.

Materials and methods

Treatments

The effect of N fertilisation on the growth and growth parameters of maize plants was studied in a small-plot long-term experiment set up on chernozem soil with forest residues by Béla Győrffy in 1961. The almost 50-year maize monoculture can be regarded as a stress environment. The N fertiliser treatments were as follows: 0, 80, 160 and 240 kg ha^{-1} (hereafter: N_0, N_{80}, N_{160}, N_{240}). All the treatments received 160 kg ha^{-1} each of P and K fertiliser. The experiment was set

up in a split-plot design with four replications, with N treatments in the main plots and maize hybrids in the subplots. The subplot size was 5.6 × 9.6 m, and sub-subplots were formed for sampling purposes. The investigations were carried out in 2001 and 2002 on three hybrids with different vegetation periods: Mv TC 272 (FAO 280), Mv 355 SC (FAO 390) and Maraton SC (FAO 450). For further details, see Berzsenyi (2009).

Sampling and measurements

Plant samples for growth analysis were taken every 14 days from the 4-leaf stage of maize (28–35 days after sowing) until physiological maturity. On each experimental plot five plants were cut off at ground level in the early stages and three plants from the 4th sampling onwards, giving a total of eight samplings each year. The leaf area per plant was determined using a Delta-T laboratory leaf area meter by recording the area of each leaf, followed by summing. The dry mass of the plants was determined after drying in a drying cabinet at 90°C for 72 hours.

Growth analysis and growth parameters

The effect of N fertilisation on the growth of maize was characterised by plant and canopy growth parameters. The following parameters were calculated for the individual plant growth: relative growth rate (RGR), net assimilation rate (NAR), leaf area ratio (LAR) and the relative leaf area growth rate (RLGR). The growth of the canopy was characterised on the basis of crop growth rate (CGR), leaf area index (LAI), harvest index (HI), leaf area duration (LAD) and biomass duration (BMD). Mean growth parameters for individual plants and the canopy were calculated for each sampling date and for the vegetative and generative growth stages. Growth parameters are discussed in detail by Evans (1972) and Hunt (1982).

Nitrogen stress (S_N) was calculated according to Greenwood (1976) as the relative reduction in the given growth parameter at N_0 compared to the same parameter at N_{240}: $S_N = 100 \times$ [(growth rate at N_{240}) – (growth rate at N_0)]/growth rate at N_{240}. Post-flowering senescence was estimated for each N treatment as the reduction in LAI at maturity relative to maximum LAI(D'Andrea et al., 2006):

Relative post-flowering LAI reduction = (maximum LAI – LAI at maturity)/maximum LAI.

Classical method of growth analysis

The classical method of growth analysis was used to calculate mean values of each growth parameter for each sampling date (Evans, 1972; Causton and Venus, 1981; Hunt, 1982). The modern software tool developed by Hunt et al. (2002) for dealing with mathematical and statistical calculations in classical plant growth analysis was used to calculate the following parameters: RGR and its components, NAR and LAR, and RLGR, together with statistical parameters (variance, confidence interval). Mean values of CGR, LAI and HI, and cumulative values of LAD and BMD were calculated according to Hunt (1982) and the data were evaluated using two-factor analysis of variance (Sváb, 1973) with the help of the GenStat 11 program.

Results and discussion

The effect of N fertilisation on the mean values of four plant growth parameters, relative growth rate (RGR), net assimilation rate (NAR), leaf area ratio (LAR) and relative leaf area growth rate (RLGR), in the vegetative and generative phases is illustrated in Tables 1 and 2 for each hybrid in each year. The seasonal dynamics of RGR, NAR and LAR revealed a gradual decline as the vegetation period proceeded, while N fertilisation caused an increase in the mean values of RGR and NAR and a decrease in that of LAR.

Table 1

Effect of N fertilisation on the mean values of relative growth rate (RGR), net assimilation rate (NAR), leaf area ratio (LAR) and relative leaf growth rate (RLGR) of different maize hybrids during the vegetative period in 2001 and 2002

Maize hybrid	2001				2002			
	N_0	N_{80}	N_{160}	N_{240}	N_0	N_{80}	N_{160}	N_{240}
	RGR (mg g^{-1} day^{-1})							
Mv 272	74.8(6.2)	76.2(5.4)	77.7(9.2)	71.6(5.7)	81.1(8.1)	86.2(6.3)	79.4(9.9)	88.8(3.2)
Mv 355	76.9(11.3)	85.0(7.0)	76.9(6.0)	74.8(8.4)	93.4(13.1)	94.7(8.0)	89.6(3.5)	87.9(7.8)
Maraton	79.1(8.5)	83.0(8.2)	74.5(8.0)	73.3(3.6)	89.6(7.6)	90.8(4.8)	91.6(6.2)	89.9(6.7)
	NAR (g m^{-2} day^{-1})							
Mv 272	10.1(1.7)	11.4(2.3)	13.1(2.4)	11.8(1.8)	11.0(1.5)	12.8(2.0)	12.7(2.5)	13.0(1.0)
Mv 355	11.5(3.3)	13.8(2.2)	12.5(2.1)	13.0(2.8)	14.0(3.6)	14.4(2.2)	13.1(1.4)	12.3(1.8)
Maraton	10.5(2.3)	13.0(2.9)	12.1(1.9)	12.4(1.2)	10.5(1.7)	12.1(1.5)	12.5(1.6)	11.4(1.9)
	LAR (cm^2 g^{-1})							
Mv 272	123.8(18.9)	115.3(20.6)	111.3(30.2)	103.8(17.1)	126.3(30.4)	118.2(17.5)	107.1(27.3)	114.9(8.3)
Mv 355	113.7(32.2)	121.1(35.7)	102.8(16.5)	103.8(24.0)	125.7(43.5)	125.2(37.0)	117.6(10.0)	113.2(24.4)
Maraton	115.8(23.2)	114.5(26.3)	100.6(32.5)	87.4(24.0)	134.2(26.2)	118.3(12.8)	118.2(19.4)	123.4(20.2)
	RLGR (mm^2 cm^{-2} day^{-1})							
Mv 272	3.06(0.33)	3.30(0.40)	3.23(0.49)	2.70(0.30)	3.00(0.81)	3.83(0.45)	3.37(0.56)	3.81(0.22)
Mv 355	3.35(0.79)	3.76(0.46)	3.72(0.44)	3.01(0.62)	3.66(0.71)	4.01(0.69)	4.14(0.26)	4.23(0.45)
Maraton	3.58(0.58)	3.83(0.59)	3.52(0.35)	3.64(0.55)	4.20(0.65)	4.47(0.39)	4.65(0.32)	4.48(0.26)

Growth parameter values are followed by standard errors in parentheses

The mean value of RGR consistently increased in response to N fertilisation during the vegetative phase up to a rate of N_{80} in both years for all the hybrids, from 82.6 mg g^{-1} day^{-1} to 86.0 mg g^{-1} day^{-1} (Table 1). Due to ontogenetic drift, the value of RGR in the generative phase was only a third or a quarter of that recorded in the vegetative phase. The RGR of the total dry matter in the generative phase and the RGR of the grain yield increased from 16.1–20.1 mg g^{-1} day^{-1} and from 31.4–39.4 mg g^{-1} day^{-1}, respectively, up to a rate of N_{160} (Table 2). The RGR values also clearly reflected the year effect, being greater during the vegetative growth stage in 2002 than in 2001, while in the generative phase the RGR of the whole plant and the grain yield were higher in 2001, when rainfall supplies were more favourable than in 2002. The mean value of NAR increased in response to N fertilisation up to N_{80}, from 11.3 to 12.9 g m^{-2} day^{-1} in the vegetative phase and from 7.0 to 8.9 g m^{-2} day^{-1} in the generative phase. N fertilisation caused a consistent decline in the mean value of LAR in the vegetative phase in both years, while in the generative phase a decrease was observed in all the N treatments in 2001 and up to N_{80} in 2002.

In response to N fertilisation there was a consistent rise in RLGR during the vegetative growth period (Table 1). Compared to the N_0 treatment, the increase in RLGR was greatest in the N_{80} treatment. The value of RLGR tended to increase with the vegetation period of the hybrids. Averaged over the three hybrids, the following mean values of RLGR were recorded (% day^{-1}): 2001: N_0: 3.33, N_{80}: 3.62, N_{160}: 3.48, N_{240}: 3.12; 2002: N_0: 3.62, N_{80}: 4.10, N_{160}: 4.05,

N_{240}: 4.17. In all cases the value of RLGR dropped to 0 on the 90–95th day after sowing, when the leaf area reached its maximum value. After flowering, the RLGR value decreased, so the values became negative. As is clear from Table 2, N fertilisation resulted in a substantial decrease in this parameter (leaf withering), with values ranging from –4.34 % day^{-1} in N_0 to –2.4 % day^{-1} in N_{240} in 2001 and from –4.44 % day^{-1} (N_0) to –3.51 % day^{-1} (N_{240}) in 2002.

In the case of canopy growth parameters, changes in the mean value of crop growth rate (CGR) as a function of N treatment and hybrid are illustrated for both years in Figure 1. N treatment (N) and hybrid (H) had a significant effect on CGR in both years, and in 2002 the N × H interaction was also significant. The smallest value of CGR was observed in the N_0 treatment, which received no N fertiliser, rising significantly up to N_{160}. The following values of CGR were recorded for each N treatment in the two years (g m^{-2} day^{-1}): 2001: N_0: 13.0, N_{80}: 19.7, N_{160}: 23.9, N_{240}: 23.7; 2002: N_0: 12.1, N_{80}: 17.1, N_{160}: 19.8, N_{240}: 19.9. The hybrids differed significantly for CGR in 2001, with the lowest value for Mv 272 and the highest for Mv 355 and Maraton, whereas in 2002 there was no difference between the CGR values of Mv 355 and Mv 272, while that of Maraton was higher (Fig. 1). The higher values of CGR recorded in 2001 could be attributed to the more favourable rainfall supplies.

Table 2

Effect of N fertilisation on the mean values of relative growth rate (RGR), net assimilation rate (NAR), leaf area ratio (LAR) and relative leaf growth rate (RLGR) of different maize hybrids during the generative period in 2001 and 2002

Maize hybrid	2001				2002			
	N_0	N_{80}	N_{160}	N_{240}	N_0	N_{80}	N_{160}	N_{240}
	RGR (mg g^{-1} day^{-1}) of total dry matter							
Mv 272	19.6(5.8)	21.3(7.3)	19.0(5.5)	19.9(3.9)	14.0(4.1)	17.8(6.3)	17.2(6.2)	19.6(5.0)
Mv 355	16.6(8.7)	22.8(4.6)	23.5(4.1)	20.9(5.4)	12.3(8.2)	14.6(5.1)	17.1(3.7)	19.7(5.3)
Maraton	15.5(6.8)	16.4(11.3)	24.5(4.7)	23.7(2.1)	18.5(5.3)	15.8(4.9)	19.4(3.3)	20.5(5.0)
	RGR (mg g^{-1} day^{-1}) of grain yield							
Mv 272	36.3(4.6)	39.4(8.8)	40.4(4.6)	39.9(3.1)	29.9(3.7)	35.8(7.5)	35.8(6.6)	34.2(7.5)
Mv 355	34.4(8.7)	45.5(3.8)	44.7(2.4)	43.1(4.1)	30.9(7.1)	37.4(5.3)	37.4(3.3)	38.7(5.7)
Maraton	25.5(7.3)	33.2(6.9)	42.0(3.4)	41.7(1.8)	31.3(5.3	31.4(3.9)	36.3(2.9)	36.3(4.4)
	NAR (g m^{-2} day^{-1})							
Mv 272	9.0(2.4)	10.6(4.3)	10.6(3.7)	11.6(2.7)	7.2(2.1)	10.2(3.5)	8.2(2.6)	8.5(3.2)
Mv 355	7.3(3.7)	11.0(2.2)	13.8(2.4)	16.1(2.2)	6.0(3.7)	8.8(3.0)	6.5(1.5)	7.4(2.3)
Maraton	5.0(2.1)	7.0(5.0)	10.6(1.5)	11.8(1.0)	7.3(1.9)	5.6(2.0)	6.5(0.9)	6.4(1.5)
	LAR (cm^2 g^{-1})							
Mv 272	30.0(5.4)	28.9(8.9)	24.6(4.4)	23.8(4.1)	28.8(3.6)	26.5(5.0)	26.6(5.1)	29.5(3.2)
Mv 355	29.4(8.7)	25.9(3.5)	27.8(4.4)	26.0(4.6)	28.8(7.9)	27.9(5.3)	31.3(3.3)	33.6(4.9)
Maraton	37.3(8.2)	31.0(8.5)	28.8(4.5)	29.6(4.9)	37.4(7.2)	35.4(4.6)	36.0(5.0)	39.6(7.2)
	RLGR (mm^2 cm^{-2} day^{-1})							
Mv 272	–3.41(0.82)	–2.72(0.85)	–1.31(1.29)	–1.24(0.84)	–4.87(1.27)	–7.06(1.29)	–3.61(1.28)	–6.07(1.19)
Mv 355	–3.76(1.12)	–5.08(2.34)	–2.22(1.05)	–2.79(2.76)	–5.29(1.50)	–3.81(2.51)	–3.37(1.85)	–3.12(1.32)
Maraton	–5.86(2.22)	–2.52(1.37)	–4.03(1.63)	–3.17(0.67)	–3.15(2.04)	–3.01(6.15)	–1.59(0.45)	–1.35(0.49)

Growth parameter values are followed by standard errors in parentheses

The dynamics of leaf area index (LAI) over time was similar to that of the leaf area per plant (Berzsenyi, 2009). The effects of N fertilisation and hybrid were characterised using the maximum value of LAI (LAI_{max}) and the cumulative values of leaf area duration (LAD) calculated from LAI. N fertilisation and hybrid had a significant effect on LAI_{max} at the P = 1% level in both years, but the N fertilisation × hybrid interaction was not significant (Fig. 1). In both years the value of LAI_{max} increased significantly in response to N fertilisation up to the N_{80} rate, after which there was no further significant change. As regards differences between the hybrids, the LAI_{max} value was lowest for Mv 272 and significantly greater for Mv 355. In 2002 the LAI_{max} value of Maraton significantly surpassed that of Mv 355, while in 2001 the difference between the two hybrids was not significant. The LAI_{max} values for each N treatment and year were as follows: in 2001: N_0: 2.48, N_{80}: 3.12, N_{160}: 3.24, N_{240}: 3.30; in 2002: N_0: 2.84, N_{80}: 3.59, N_{160}: 3.64, N_{240}: 3.72. The LAI_{max} values for the hybrids in each year were: in 2001: Mv 272: 2.82, Mv 355: 3.04, Maraton: 3.24; in 2002: Mv 272: 3.04, Mv 355: 3.33, Maraton: 3.97.

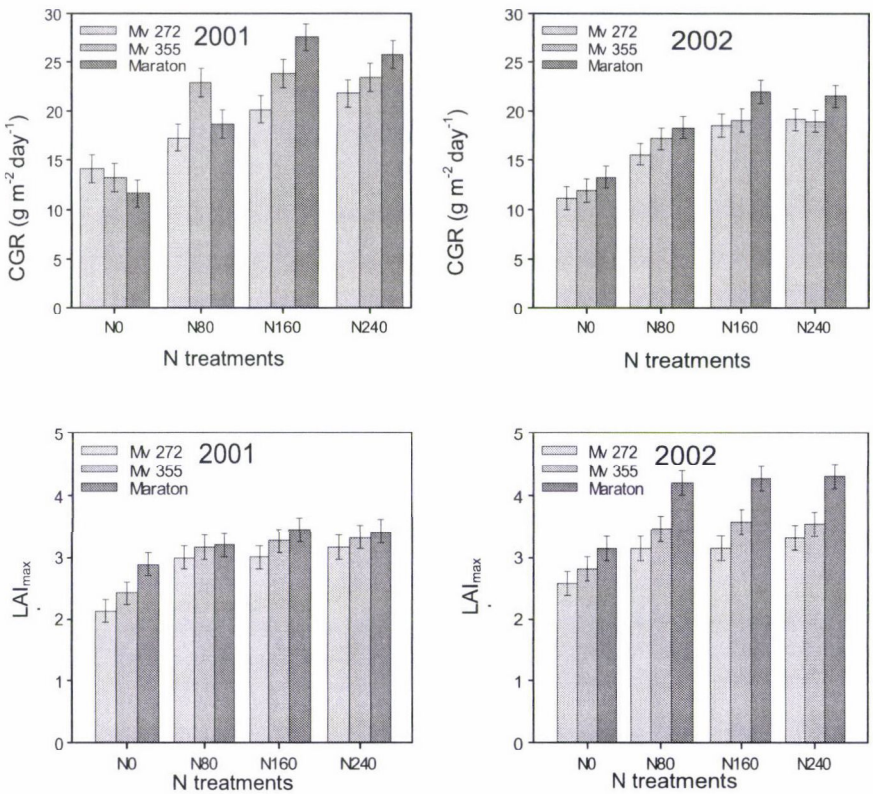

Fig. 1. Effect of N fertilisation on the mean value of crop growth rate (CGR) and the maximum value of leaf area index (LAI_{max}) of different maize hybrids in 2001 and 2002. Vertical bars indicate the standard errors of the means

Changes in the harvest index (HI) in response to N fertilisation and hybrid are illustrated in Figure 2. In both years the effect of N fertilisation and hybrid on HI was significant at the P = 0.1% level. In 2001 the N × H interaction was also significant. In response to N fertiliser the value of HI was the lowest at N_0 and rose significantly in the N_{80} treatment, while higher N rates caused no significant increase. Averaged over the two years, the HI values in the various N treatments were as follows (%): N_0: 46.9, N_{80}: 53.7, N_{160}: 53.3, N_{240}: 54.4. In both years the HI value of Mv 355 was significantly the highest (54.9%), followed by Maraton (51.2%) and Mv 272 (50.1%). Among the two years, the HI values in 2001 were significantly higher than in 2002 (55.1 vs. 49.0%).

The cumulative effect of N fertilisation and hybrid on leaf area duration (LAD) and biomass duration (BMD) is illustrated in Figure 2. Both N fertilisation and hybrid had a significant effect on LAD in both years, while the N × H interaction was also significant at the P = 5% level in 2001. In both years the effect of N fertilisation on LAD increased significantly up to N_{160}, after which no further significant change was observed. The greatest difference in LAD values was observed between the N_0 and N_{80} fertiliser rates. The annual values of LAD for each N treatment were as follows (LAD day): in 2001: N_0: 180.1, N_{80}: 236.9, N_{160}: 268.2, N_{240}: 261.0; in 2002: N_0: 162.0, N_{80}: 230.9, N_{160}: 265.5, N_{240}: 264.1. In both years the value of LAD was found to be correlated with the vegetation period of the hybrids, with significant differences between the values. The LAD values recorded for each hybrid in each year were as follows (LAD day): in 2001: Mv 272: 210.9, Mv 355: 236.6, Maraton: 262.2; in 2001: Mv 272: 195.2, Mv 355: 222.0, Maraton: 274.7.

Both N fertilisation and hybrid had a significant effect on the cumulative value of biomass duration (BMD) (Fig. 2). In both years BMD increased significantly up to the N_{160} rate, giving the following values (10^3 g day) in the various treatments: in 2001: N_0: 9.49, N_{80}: 13.12, N_{160}: 15.79, N_{240}: 15.50; in 2002: N_0: 7.99, N_{80}: 10.96, N_{160}: 12.40, N_{240}: 11.81. The value of BMD increased with the vegetation period of the hybrids in both years, giving the following values (10^3 g day) for the individual hybrids: in 2001: Mv 272: 12.5, Mv 355: 13.92, Maraton: 13.99; in 2002: Mv 272: 10.42, Mv 355: 10.50, Maraton: 11.45. It is clear from the data that BMD had higher values in the favourable year of 2001 than in 2002. Averaged over the treatments, the value of BMD was 13.47 10^3 g day in 2001 and 10.79 10^3 g day in 2002.

Among the canopy growth parameters, the mean values of CGR and the cumulative values of LAD and BMD were used to quantify the extent of nitrogen stress (S_N), based on values recorded in the N_0 and N_{240} treatments. All three parameters demonstrated similar stress effects (29.4–38.3%) in the N_0 treatment. The following S_N values (%) were recorded for each growth parameter, averaged over the hybrids: based on CGR: in 2001: 29.4, in 2002: 32.9; based on LAD: in 2001: 30.9, in 2002: 38.3; based on BMD: in 2001: 36.7, in 2002: 32.6. The relative decline in LAI after flowering was also calculated for

the individual N treatments. Averaged over the hybrids the following values (%) were obtained: in 2001: N_0: 70.1, N_{80}: 54.5, N_{160}: 45.2, N_{240}: 43.2; in 2002: N_0: 78.3, N_{80}: 72.7, N_{160}: 54.5, N_{240}: 52.3. The relative decrease in LAI after flowering was found to be 16–27% less severe in 2001 and 6–26% less severe in 2002.

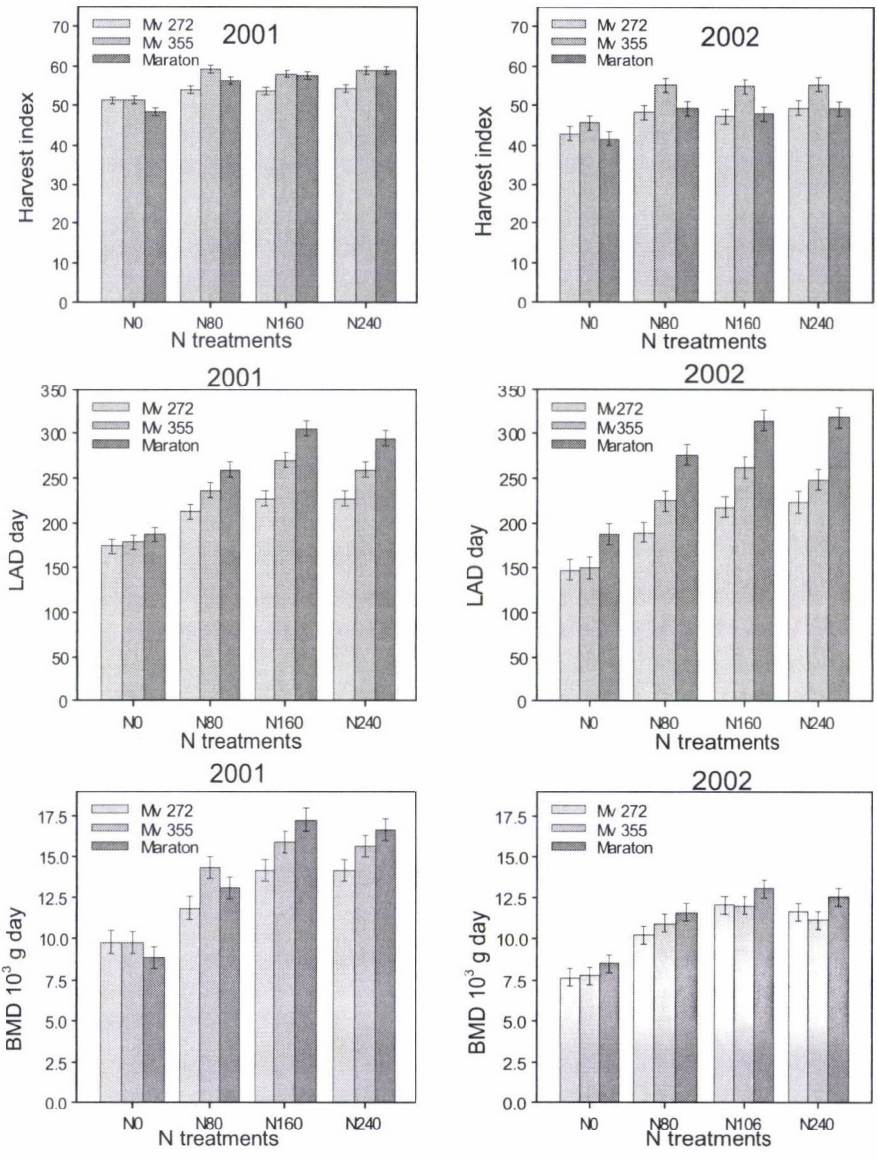

Fig. 2. Effect of N fertilisation on the harvest index, cumulated value of leaf area duration (LAD) and biomass duration (BMD) of different maize hybrids in 2001 and 2002. Vertical bars indicate the standard errors of the means

Conclusions

N fertilisation was demonstrated to have a significant effect on the dynamics of both the dry matter accumulation and leaf area of maize plants (Berzsenyi, 2009) in terms of both individual plant and canopy growth parameters. The growth parameters of the individual plants gave a good indication of N fertiliser effects. RGR and NAR increased, while LAR decreased with a rise in the N rate, during both the vegetative and generative phases. N fertilisation was found both to increase the relative growth rate of the leaf area and to reduce the rate of leaf withering after flowering. The results show that with the growth analysis software reported by Hunt et al. (2002) the classical method of growth analysis can be used to calculate both the mean values of the parameters and also the statistical parameters, thus allowing significant treatment effects to be detected. Without this software these calculations may be extremely tedious, so the statistical parameters are frequently omitted.

The effects of both N fertilisation and hybrid could be satisfactorily described using the growth parameters of the canopy. The rise in CGR in response to N fertilisation was correlated chiefly with greater LAI and to a lesser extent with an increase in NAR. LAI was severely affected by low N availability, mainly because of reduced leaf expansion before silking and accelerated leaf senescence from silking onward. Hybrids with longer vegetation periods had higher values of CGR, LAI_{max} and cumulative LAD and BMD. The integrated LAD and BMD parameters give an exact picture of the size and duration of the leaf area and dry matter production at any given time, thus providing a reliable expression of the effects of N fertilisation and hybrid. In conformity with international research (Tollenaar et al., 1993; Sinclair, 1998), the present results suggest that HI is a relatively stable parameter, changing to a lesser extent in response to environmental and agronomic factors than the biomass production per plant or the grain yield. The experimental data demonstrated a year effect, which influenced the temporal dynamics of the N fertiliser response of maize hybrids. The canopy growth parameters (CGR, LAI, LAD, BMD) gave a good characterisation of how N stress affected maize growth. It could be concluded from the results that knowledge on the dynamics of dry matter accumulation and on growth parameters could contribute to an understanding of changes in the N fertiliser response over time and to improvements in the N utilisation of maize hybrids.

Acknowledgements

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COMPARATIVE ANALYSIS OF LEAFY AND NON-LEAFY SILAGE MAIZE HYBRIDS

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Twelve silage hybrids were included in field experiments in Martonvásár in 2007 and 2008 to compare the agronomic traits and chemical quality traits of leafy and non-leafy hybrids. The climatic data for the two experimental years differed considerably. The results reflected the differences in weather conditions. Thanks to the plentiful rainfall in 2008 the hybrids reached their genetically determined height (274.32 cm on average), while in 2007 the average height was only 238.03 cm. In both years a leafy hybrid was the tallest, while the shortest plants were non-leafy. The assimilation leaf area above the main ear was greatest for the five leafy hybrids in both years, with values of 0.35–0.45 m^2 per plant for conventional hybrids and 0.53–0.84 m^2 per plant for leafy hybrids, averaged over the two years. The larger leaf area in leafy hybrids could be attributed both to the larger number of leaves and to the fact that they were broader. The greatest ear mass per plant was produced by Mv Massil (198.66; 320.00 g), a leafy hybrid which also had the greatest leaf area above the main ear. In addition to large green mass (leaf, stalk), an ideal silage maize hybrid should also have satisfactory grain yield. Several of the leafy and non-leafy hybrids in the experiment gave favourable results. In the present experiment the highest starch content was recorded for a leafy hybrid, while the highest protein and oil contents were characteristic of early maturing, non-leafy hybrids. Nevertheless, three of the leafy hybrids had above-average protein content.

Key words: silage hybrids, leafy, non-leafy, agronomic traits, chemical quality

Introduction

The silage maize hybrids bred in Martonvásár are generally characterised by high yield potential (the ear makes up a large proportion of the total dry matter), valuable chemical components and good digestibility. In addition to conventional silage hybrids, a number of leafy hybrids from Martonvásár have also been state registered. The first leafy silage maize hybrid in Europe was developed in Martonvásár and registered under the name Kámasil in 2002.

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The main characteristic of leafy hybrids is that they have more leaves than normal hybrids. The presence of the dominant *Lfy1* gene transforms the plant architecture by increasing the number of leaves above the ear, which are important for photosynthesis. In these hybrids the main ear is attached lower down the stalk, the internodes are shorter, the stalk lignin content is greater and the plants have greater yield potential. The *Lfy1* gene was first described in detail by Shaver (1983), who reported on the origin and inheritance of the gene and on its generally positive effect on the morphology and yield of maize.

A larger number of leaves above the ear results in a greater assimilation leaf area (Pintér et al., 2003), allowing the plant to bind the light energy required for photosynthesis more efficiently and resulting in the production of more nutrients in the leaves (Dwyer et al., 1995). The leaves above the ear are important for grain filling and yield, as they are younger, photosynthesise more actively and transport nutrients into the grain more easily than the older leaves below the ear (Subedi and Ma, 2005). Due to the larger leaf area above the ear, the vegetative period of leafy genotypes is shorter and the grain-filling period longer (Begna et al., 2001; Modarres et al., 1997). This has a positive effect both on the yield and on grain quality, as reported by a number of authors (Stewart and Dwyer, 1993; Begna et al., 2001; Modarres et al., 1997; Dijak et al., 1999). The chemical quality and digestibility of the silage is just as important as the achievement of high fresh and dry matter yields per hectare. The starch content, the quantity and quality (amino acid composition) of the protein, the digestible fat content, the fibre and lignin contents, the grain/stalk and grain/leaf ratios, the stay-green trait and softer seed coats are all factors that influence silage quality and digestibility. Leafy hybrids represent a leap in quality, as they have not only favourable quality traits but also high yields. The greater ratio of the leaves in the total plant dry matter and the higher carbohydrate content of the leaves above the ear (Andrews et al., 2000) also have a beneficial effect on silage quality and digestibility. It was previously thought that only a high grain ratio was favourable for digestibility, as the most valuable nutrients are accumulated in the grain. In Hungary, too, only this trait was analysed when registering silage maize hybrids. In the case of leafy hybrids, however, there is also a high ratio of assimilates in the increased number of leaves above the ear, and these are present in a form just as easily digested as those in the grain (Perry and Caldwell, 1969). In other words, the greater leaf area above the ear results in better quality silage. Several authors (Bal et al., 2000; Clark et al., 2002; Thomas et al., 2001; Benefield et al., 2006) have shown that feeding silage made from leafy hybrids to dairy cows increases the dry matter uptake and the quantity of *in vitro* digestible dry matter and improves the digestibility of starch and fibre (NDF = neutral detergent fibre), causing the cows to give larger quantities of milk with a higher protein content than when they are fed silage from normal hybrids. The cultivation of leafy hybrids for silage production is thus advantageous from numerous points of view.

Materials and methods

An experiment on leafy and non-leafy silage hybrids (Table 1) was set up with four replications in Martonvásár in 2007 and 2008.

Table 1
Leafy and non-leafy silage maize hybrids tested in the experiment

Leafy silage hybrids	FAO number	Non-leafy silage hybrids	FAO number
Limasil	380	Mv 241	260
Dunasil	390	Mv 298	300
Kámasil	510	Mv 352	330
Mv 504	580	Maros	330
Mv Massil	610	Mv NK 333	390
		Mv TC 434	440
		Maxima	580

A comparison was made of the agronomic traits (plant height, main ear attachment height, leaf area above the main ear, ear mass per plant, main ear length, thousand-kernel mass) and chemical quality traits (starch, protein and oil content) of conventional and leafy silage maize hybrids.

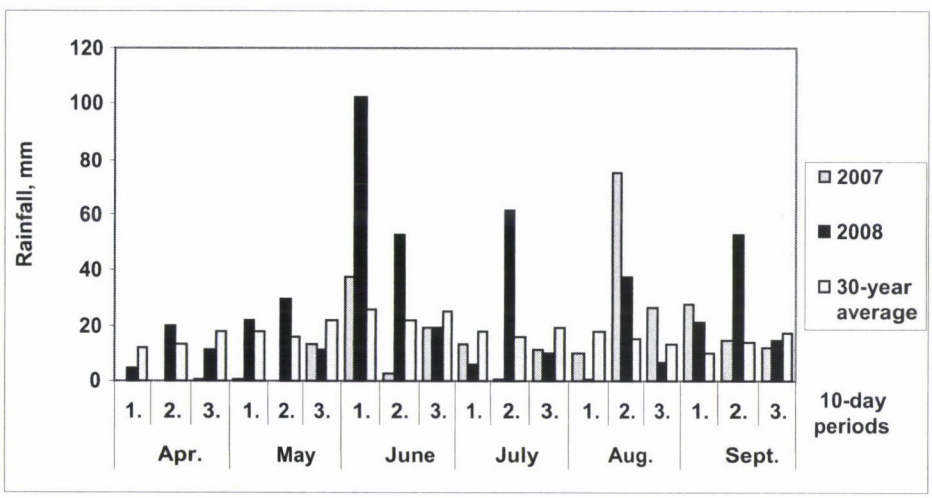

Fig. 1. Rainfall quantities during the vegetation period in the experimental years (mm)

The weather in the two experimental years differed considerably, with only 265 mm rainfall in 2007, 47 mm less than the mean over many years (Fig. 1), while 2008 was ideal from this point of view, with 482.7 mm rainfall, 170.7 mm more than the long-term mean. There was little difference in the mean temperature of the two years (2007: 18.2°C, 2008: 18.0°C, long-term mean: 17.7°C). While the total rainfall in 2007 was less than the long-term mean, the number of very hot days during the vegetation period was 58, compared with a long-term mean of 39. During the most critical period, at flowering, the maximum daytime temperature was above 30°C for 20 consecutive days, and the plants suffered from a long period of atmospheric drought. There were fewer very hot days in 2008 (42), and during flowering in July the maximum daytime temperature was only above 30°C for 14 days.

Results

Plant height and attachment height of main ear

The mean height of the hybrids included in the experiment was 238.03 cm in 2007 and 274.32 cm in 2008 (Fig. 2). The plentiful rainfall in 2008 allowed the plants to reach their genetically determined height, while the drought in 2007 resulted in a mean height of 234.02 cm for non-leafy hybrids and 243.63 cm for leafy hybrids. In 2008, thanks to the large amount of rainfall during the growth period (May, June) an ideal height was achieved by both the leafy (283.92 cm) and non-leafy (267.46 cm) hybrids. The plant height and the attachment height of the main ear are closely correlated traits. The ear attachment height averaged 103.73 cm in 2007 and 112.18 cm in 2008. As also reported in the literature, the main ears of leafy hybrids were attached lower in both years (102.90; 111.63 cm) than those of non-leafy hybrids (104.32; 113.58 cm). These differences were statistically significant.

Leaf area per plant above the main ear

In both years the leaf area above the main ear was greater for leafy hybrids (non-leafy: 0.41 m^2 per plant, leafy: 0.70 m^2 per plant, averaged over the two years; Fig. 3). This larger assimilation area was due to the greater number of leaves above the main ear (leafy: 9.90, non-leafy: 6.37) and to the greater width of the leaves (leafy: 10.51 cm, non-leafy: 9.35 cm). Averaged over two years, the young, actively photosynthesising leaf area above the main ear ranged from 0.35–0.45 m^2 per plant for non-leafy hybrids and from 0.53–0.84 m^2 per plant for leafy hybrids.

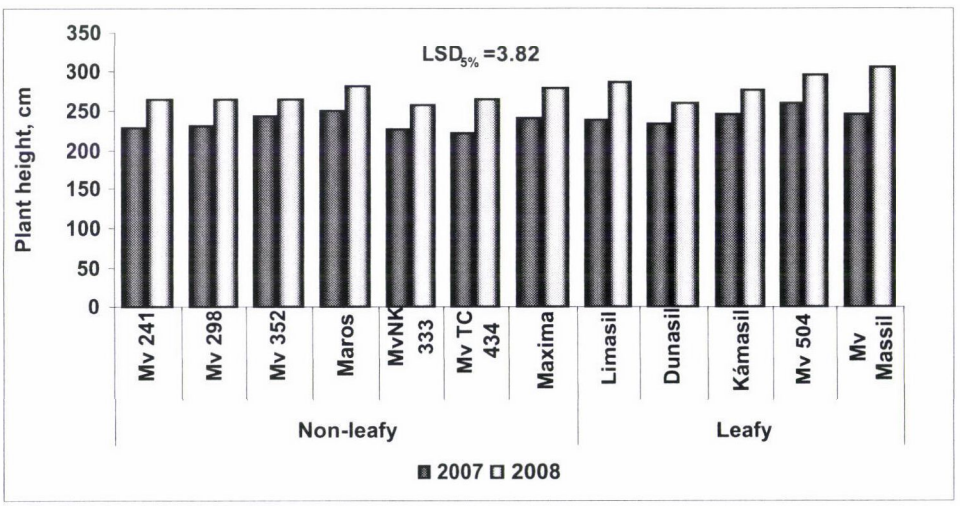

Fig. 2. Plant height (cm) of leafy and non-leafy silage hybrids in the experimental years

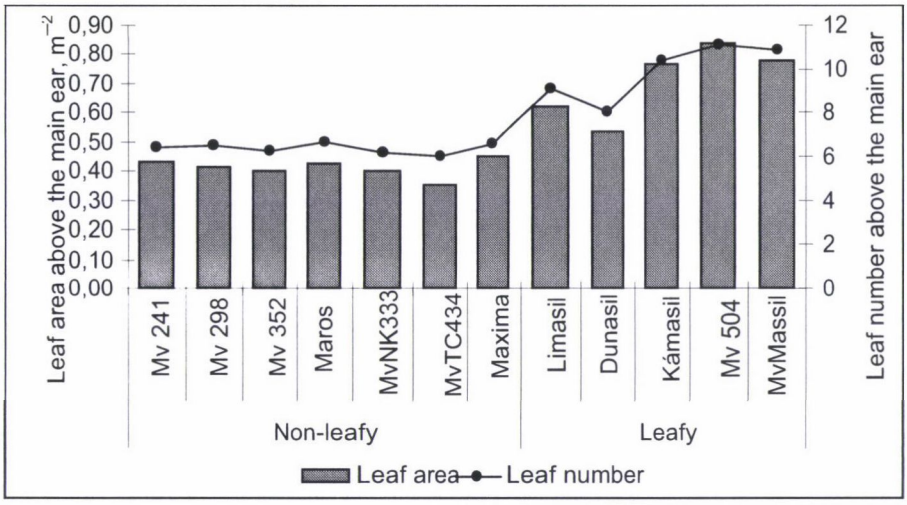

Fig. 3. Leaf area (m² per plant) and leaf number above the main ear in leafy and non-leafy hybrids, averaged over 2007 and 2008

Ear mass per plant

For the hybrids investigated, the ear mass per plant was considerably modified by the year, with mean values of 167.76 g in 2007 and 287.33 g in 2008. The good results achieved in 2008 could be mainly attributed to the quantity of rainfall, which amounted to 73.4 mm prior to flowering (May–June) in 2007 and to 236.6 mm in 2008. During the flowering period (July) these figures were 25 mm in 2007 and 76.8 mm in 2008. The low rainfall sum was associated with atmospheric drought in 2007, with maximum daytime temperatures of over 30°C for 20 consecutive days, as compared to 14 in 2008. The highest ear mass per plant was observed for leafy hybrids in both years (174.57 g; 306.00 g), with significantly lower values for non-leafy hybrids (162.90 g; 274.00 g) (Table 2). In both years the higher yields could be attributed primarily to greater thousand-kernel mass and longer ears. Averaged over the two years the thousand-kernel mass was 353.84 g for leafy hybrids and 338.60 g for non-leafy hybrid, while the main ear length was 19.17 cm for leafy and 18.64 cm for non-leafy hybrids. No significant difference was found between the two silage hybrid types for the number of ears per plant (leafy: 1.96, non-leafy: 1.90).

Table 2
Yield per plant and yield components of leafy and non-leafy hybrids, 2007–2008

Traits	2007		2008		Average	
	Leafy	Non-leafy	Leafy	Non-leafy	Leafy	Non-leafy
Ear mass per plant, g	174.57	162.90	306.00	274.00	240.28	218.45
Ear length, cm	16.70	17.25	21.64	20.03	19.17	18.64
1000-kernel mass, g	347.05	328.32	360.63	348.88	353.84	338.60
Ear per plant	1.80	1.80	1.96	1.90	1.88	1.85

Grain quality

The starch content of the grain was closely correlated to the yield per plant. In response to the higher rainfall sum in 2008 there was greater starch incorporation into the kernels than in the dry year of 2007. In both years the starch content was higher in leafy hybrids (70.36; 72.42%) than in non-leafy hybrids (69.25; 71.71%). Averaged over the two years the highest starch contents were observed for Maxima (71.79%), among the non-leafy hybrids, and Mv Siloking (72.24%), among the leafy hybrids. The protein content of the grain yield, in contrast to the starch content, was higher in the dry year (9.85%) and lower in the wet year (8.71%). In both years higher protein contents were recorded for non-leafy hybrids (10.04; 8.98%) than for leafy hybrids (9.58; 8.34%). The Bravais correlation coefficient between the two parameters was –0.67 (Fig. 4). In the dry year (2007) the kernel oil content was higher (4.08%) than in the wet year (2008; 3.11%). In 2007 non-leafy hybrids had greater oil content (3.29%) than leafy hybrids (2.86%), while in 2008 there was no significant difference between the two hybrid types (4.07; 4.08%). The trend in oil content was similar to that of protein content, with a Bravais correlation coefficient of 0.66 between the two parameters.

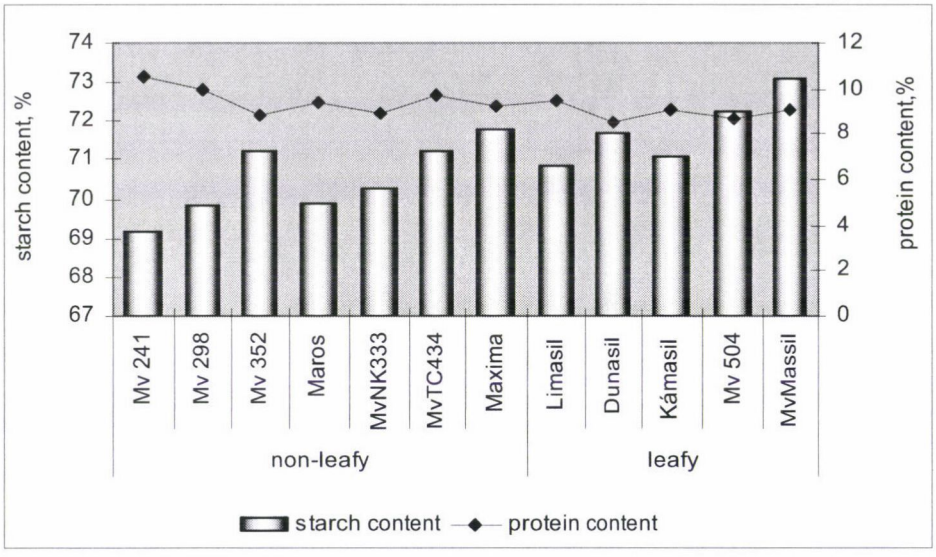

Fig. 4. Starch and protein contents of leafy and non-leafy hybrids, averaged over 2007 and 2008

Conclusions

A comparison was made of the agronomic and quality traits of conventional (non-leafy) and leafy silage maize hybrids. As reported by other authors (Shaver, 1983), the leafy hybrids grew taller than the conventional silage

hybrids and had a larger leaf area above the main ear, which could be contributed to the larger number of leaves and the greater leaf width. In the present experiment, the main ears of the leafy hybrids were attached lower than those of non-leafy hybrids. Thanks to the larger assimilation surface above the main ear, the leafy hybrids had greater yield per plant, primarily due to the longer ears and higher thousand-kernel mass (Steward and Dwyer, 1993; Begna et al., 2001). The kernels of leafy hybrids had greater starch content than those of non-leafy hybrids. As found in earlier work (Hegyi et al., 2001), the protein and oil contents of the grain were higher in the dry year (2007), while starch incorporation was greater in the wet year (2008).

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PRODUCTIVITY AND NITROGEN USE OF MAIZE AS AFFECTED BY *IN SITU* AND *EX SITU* GREEN MANURING IN MAJOR AND MINOR SEASONS OF TROPICAL ASIA

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Green manuring is considered an important agronomic practice for smallholder farming systems in the tropics. Different species of legumes and non-legumes are applied either as *ex situ* or *in situ* green manures. Thus a field study conducted under rainfed conditions in Sri Lanka compared the effect of *in situ* and *ex situ* green manuring using two popular green manures (*Crotolaria juncea* – a legume and *Tithonia diversifolia* – a non-legume) on the growth, seed yield and N use pattern of maize (*Zea mays*), the most popular upland cereal in the Asian tropics, grown with high and low N rates, in the two seasons that correspond to the monsoonal rains. *In situ* green manuring, especially with crotolaria, increased the growth, seed yield and N use efficiency of maize when compared to the *ex situ* addition of similar quantities of the green manure. The impact was also greater in the minor season, when the crop was subjected to moisture stress conditions. The benefits of *in situ* green manuring with tithonia were lower than those due to crotolaria. *In situ* green manuring with tithonia also led to a greater increase in growth, seed yield and N use efficiency in maize compared to *ex situ* green manuring with this species. The green manures, especially *in situ* application, also increased the benefits of enhanced rates of N fertilizer to the crops in both seasons, with greater use efficiency in the major season. The study showed the benefits of green manuring (both *in* and *ex situ*) for obtaining higher yields of maize in the growing seasons of the Asian tropics, especially the *in situ* application of legume species.

Key words: green manuring, *in situ* and *ex situ* applications, maize, humid tropics

Introduction

The rapid decline in soil fertility and the high costs of fertilizer are the principal factors limiting food production in the developing nations of the tropics. Due to the increasing population pressure and demands on land use for food production, fallow period are not feasible. Furthermore, nitrogen (N) is considered to be one of the most limiting factors of tropical crop production, especially for N-demanding crops such as cereals (Moser et al., 2006; Nakhone and Tabatabai,

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2008). N is the most extensively used fertilizer in the developing nations of the tropics due to the rapid visible impact when applied to crops and its low availability in tropical soils (Barker and Pilbeam, 2006). Therefore, the efficient management of soil fertility and applied N becomes important for successful tropical cereal production (Ladha et al., 2005) to ensure high yields and for environmental sustainability by reducing gaseous and leaching losses.

Green manuring is considered a useful management practice in all agricultural systems of the tropics due to the ability of these crops to enhance sustainability by reducing erosion and promoting the development of soil properties (Dinnes et al., 2002), while also possibly reducing the problems caused by global warming (Robertson et al., 2000; Tejada et al., 2008). Thus many types of species, including both leguminous and non-leguminous plants, are used for green manuring in the tropics. Legumes such as *Crotalaria juncea* are important due to their ability to fix atmospheric N and the production of large quantities of biomass with a low C:N ratio (Sharma, 2004), while non-legume species such as *Tithonia diversifolia* help the supply of phosphorus (P) to subsequent crops (Jama et al., 2000; Chukwuka and Omotayo, 2008). Both legumes and non-legumes have been used successfully to enhance the growth and yields of tropical species, especially maize (*Zea mays*) (Sangakkara et al., 2008), when incorporated into soils in the minor season of the tropics, when the crops are subjected to soil moisture stress. In addition, the allocation of green manures also enhances the benefits of added fertilizers in terms of increased uptake (Sakala et al., 2003) due to the ability of the organic matter to retain nutrients in the rhizosphere (Vanlauwe et al., 2002). Thus, green manures could help tropical smallholders to maintain soil fertility and use added nutrients, especially mobile elements such as N, more efficiently for successful crop production.

Green manures are either applied after being grown *in situ* during fallow periods, after harvest, or from external sources, when it is referred to as *ex situ* manuring (Aulakh and Grant, 2008). Most research highlights the importance of different green manures grown *in situ* on the growth of a wide range of tropical crops (e.g. Perin et al., 2006; Choi et al., 2008; Tejada et al., 2008; Sangakkara and Stamp, 2008; Sincik et al., 2008). Furthermore, Kaewpradit et al. (2009) reported the increased N use efficiency of rice due to the application of ground residues as a green manure with straw. Other research projects cite the usefulness of the *ex situ* application of green manures for tropical crops (e.g. Gachengo et al., 1998; Bah and Rahman, 2001). In contrast, a comparative analysis of the impact of the *in situ* and *ex situ* application of green manures on the growth and yields of tropical crops and on nutrient use efficiencies has not been clearly presented. Thus, field studies were carried out in Sri Lanka over the major and minor seasons of Asia, using maize as a test crop to determine (i) the impact of applying similar rates of green manures grown *in situ* or as *ex situ* material on the growth and yields of maize, and (ii) the N harvest indices and N use efficiencies of applied fertilizer. The green manure crops used were *Crotalaria juncea*, a popular leguminous species, and *Tithonia diversifolia,* a common non-legume species.

Materials and methods

The experiments were conducted on two sites located 200 m from each other to overcome the carry-over effects of the treatments from the major season to the minor season. The period of study was from October 2005 – February 2006, to cover the major wet season, and from late April – August 2006, to encompass the minor dry season. The location was a farm in close proximity to the University Experimental Station (8°N, 81°E, and 420 m above sea level). The soil of the site is an Ultisol (Rhododult) with a mean depth of 1.25 m (Panabokke, 1996). The texture of the soil was sandy loam, with a pH (1:2.5 H_2O) of 6.46 (± 0.51) and total N, available P and K contents of 29 (± 2.05) mg g^{-1}, 6.8 (± 0.31) mg g^{-1} and 10.1 (± 0.98) mg g^{-1}, respectively. The CEC of the soil was 29.8 ± 1.22 meq. 100 g^{-1} soil and the organic matter content was 1.29 ± 0.19 g kg^{-1} soil.

The mean climatic parameters of the experimental seasons were as follows: In the major wet season (October – February), the rainfall received was 1124 mm, while the mean daily temperature was 28.6 ± 2.04°C, with a mean daily humidity of 84% ± 2.8% and a mean pan evaporation of 2.59 ± 0.34 mm day^{-1}. In the minor season (April – August), which is warmer and drier, generally subjecting the crops to water stress if cultivated under rainfed conditions, the rainfall received was 398 mm, with a mean daily temperature of 32.1 ± 1.99°C, mean daily humidity of 76% ± 3.2% and a mean pan evaporation of 4.01 ± 0.52 mm day^{-1}.

The treatments applied in the experiments in both seasons were as follows: The green manures selected were *Crotalaria juncea* and *Tithonia diversifolia,* applied either as *in situ* or *ex situ* mulches, with two N regimes (25 or 50 kg N per ha), with control plots where only the two rates of N were applied. Thus, in each season there were 10 plots replicated three times within a factorial experiment using a randomized block design.

In the first weeks of August 2005 and March 2006, 30 plots of 5 × 4 m were prepared and on the randomly determined plots to which the *in situ* mulches were to be applied, either seeds of crotalaria were broadcast or uniform cuttings (5 cm) of tithonia were planted at a spacing of 30 × 30 cm. At the same time, the same species were planted on an adjacent site to procure the biomass for *ex situ* mulching.

With the onset of the first rains in the two seasons (last week of September and mid-April for the major and minor seasons, respectively), the biomass of the green manures was measured within a quadrat of 1 × 1 metre by uprooting the plants and measuring the fresh weights after removing the soil. Thereafter the green manures were incorporated into the respective plots. At the same time, similar quantities of biomass (both shoots and roots) of the two green manures were removed from the external source and applied to the predetermined plots and incorporated as *ex situ* mulches. The plots receiving only fertilizers were also prepared and kept weed-free. At 14 days after incorporation, maize (*Zea mays*, open-pollinated variety Ruwan, germination 92%) was planted in both seasons at the recommended spacing of 60 × 30 cm (Department of Agriculture, 1989). The recommended fertilizer rate was applied, with the equivalent of 45 kg P and 30 kg K at planting. The N treatments of 25 or 50 kg N were applied in two split applications at planting and at 45 days after planting. Weeding was carried out manually on two occasions.

In each season, the following measurements were made:

Total biomass of *in situ* mulches prior to incorporation to determine the quantity to be added from *ex situ* sources, and the organic C (Walkley and Black method – Walkley and Black, 1934) and total N contents (Kjeldhal method – Jackson, 1958) of both *in situ* and *ex situ* biomass to determine the C:N ratios;

Shoot dry weight (drying at 80°C for 48 hours) of 4 plants per plot, taken at 10-day intervals until anthesis for calculating the relative growth rate (RGR) as described by Hunt (1982), and determining the mean RGR over the vegetative growth period;

At the onset of anthesis, the SPAD values (SPAD Meter, Minolta) of the two topmost fully opened leaves were recorded between 0800 and 1000 hrs on 4 points per leaf;

Days to 50% anthesis;

At crop maturity, seed and stover yields were recorded to determine harvest indices;

The N contents of the whole plants at anthesis and of the seeds and stover at harvest were determined using the Kjeldhal method to calculate the following:

N harvest index (NHI) = Ratio of N content in grain:N content in stover (inclusive of roots) at harvest

N use efficiency (NUE) % = Ratio of yield:total N uptake by the plant × 100

The data of the respective seasons were subjected to analysis of variance using the general linear model (GLM) of the SAS statistical package (SAS, 1994). Tukey's LSD test was used to separate means when the F test was significant (P=0.05), using Arc sin transformations when necessary to ensure normal distribution of the data.

Results and discussion

Green manures

The total shoot and root biomass produced by crotalaria and tithonia at the time of incorporation, when planted *in situ*, were 402 ± 24 g m^{-2} and 506 ± 18 g m^{-2}, respectively, in the major season and 379 ± 14 g m^{-2} and 405 ± 20g m^{-2} in the minor season, respectively. This implied that tithonia has the potential to produce greater biomass than crotalaria over 40–45 days, and that the productivity of both species was higher in the period just prior to the major season, due to the more favourable climate in terms of rainfall received. However, the C:N ratios of the two green manures did not vary between seasons and were 12.42 ± 1.46 and 17.07 ± 2.01 in crotalaria and tithonia, respectively, under both *in situ* and *ex situ* conditions, illustrating that these chemical properties of the two green manures are not affected by the location of planting. Furthermore, the addition of green manures under *ex situ* or *in situ* conditions did not affect the quantity or quality of the material, thus reducing the experimental error.

Vegetative growth of maize

In both seasons, the three-factor interactions between the green manures, methods of addition and rates of N fertilizer application were not significant (Table 1). All the two-factor interactions were significant in terms of RGR, SPAD and days to 50% anthesis of maize in both seasons, indicating the effects of different treatments on the vegetative growth of this important tropical cereal. The seasonal effect was not measured, as the trial was not replicated.

In general, the vegetative growth of maize was lower in the minor season, irrespective of the two green manures, due to the more adverse climatic conditions caused by lower rainfall and higher temperatures and evaporation rates. Most rainfed crops are adversely affected in this season, and hence vegetative growth is retarded, while the plants produce flowers earlier, as seen in days to 50% anthesis. This could be attributed to the early maturing of maize under higher temperature and lower moisture conditions (Soler et al., 2007).

The use of crotalaria as a green manure increased the RGR and SPAD values of maize to a greater extent in both seasons, although the impact was more evident in the minor season. In contrast, the application of crotalaria delayed the days to anthesis, which could be attributed to the greater N content

of the leguminous green manure, as shown by its lower C:N ratio. In contrast, although the application of tithonia as a green manure enhanced the RGR and SPAD values over those of the control plots which received only fertilizers, this green manure did not stimulate vegetative growth to the same extent as crotalaria, due to the higher C:N ratio. However, the benefits of the two green manures were clearly evident in terms of enhancing the growth of maize, thus indicating their benefits for this important tropical highland cereal, as reported earlier (e.g. Gachengo et al., 1998; Ademiluyi and Omotoso, 2007; Sangakkara and Stamp, 2008).

In situ green manuring had a greater beneficial impact on the vegetative growth of maize, although the same quantities were applied from *ex situ* sources. The benefits were greater with crotalaria, especially in the drier minor season. This highlighted the usefulness of *in situ* green manuring compared with applying the same quantity from external sources. Although the causal phenomenon was not evaluated, organic matter addition through decayed leaves and roots and the roots remaining in the soil, and the beneficial effect of root growth on the soil could have been responsible (Aulakh and Grant, 2008). This question needs further elucidation.

As expected, the application of fertilizer N increased the vegetative growth of maize and the benefits were greater with the green manures, although the days to anthesis were prolonged. The greater benefits of N fertilizer when combined with green manures are due to synergistic effects, as the organic matter helps to retain the applied fertilizer in the rhizosphere (Vanlauwe et al., 2001; Tejada et al., 2008). The benefits of added fertilizer N, including the differences between the two rates, were greater in the major season, as the crops were affected by the dry conditions in the minor season. The benefits of adding fertilizer N were also more pronounced with *in situ* green manures when compared to *ex situ* green manures, a phenomenon not clearly identified earlier and which warrants further study.

Seed yields and harvest indices

As in the case of vegetative growth, the seed yields of maize were higher in the major season due to the more conducive climate with adequate rainfall. However, in both seasons, crotalaria had the greatest beneficial impact on seed yields, compared with both the control treatment and the yields obtained with tithonia. The beneficial impact of the green manures was also greater in the minor season (Table 2), with an increase of 31% in the mean yield of maize compared to a 21% increase with tithonia over that of the control plots in the minor season. The increments in seed yields due to crotalaria and tithonia over the control plots in the major season were significantly lower (9% and 7%, respectively). This clearly implied the benefits of green manuring especially in the drier minor season, as it helps soil moisture conservation, while supplying nutrients (Tejada et al., 2008). A comparison of the two green manures illustrated that the seed yields of maize due to crotalaria were greater than those

obtained with tithonia, the benefits being greater in the minor season (2% and 8% in the major and minor seasons, respectively). This highlights the beneficial impact of a leguminous green manure due to its greater N content, which is generally the most limiting nutrient in smallholder systems in the tropics, and to the lower C:N ratios, which makes the N more easily available.

Table 1
Growth of maize plants as affected by green manure application and nitrogen fertilizers (kg ha^{-1}) over major and minor seasons

Green manure	Addition	Fertilizer N	RGR (mg g^{-1} day^{-1})	SPAD	Days to anthesis
Major season					
Crotalaria	*In situ*	25	44.1	51	50
		50	49.6	58	55
	Ex situ	25	43.2	41	48
		50	45.9	47	51
Tithonia	*In situ*	25	38.1	42	46
		50	41.7	46	49
	Ex situ	25	35.7	40	45
		50	37.9	43	48
None		25	31.4	37	43
		50	34.4	40	46
Probability					
Green manure			0.014	0.006	0.043
Addition			0.034	0.018	0.037
Fertilizer N			0.004	0.033	0.009
Green manure × addition			0.043	0.039	0.017
Green manure × N			0.007	0.024	0.039
Addition × N			0.048	0.030	0.027
Manure × addition × N			0.085	0.094	0.124
Minor season					
Crotalaria	*In situ*	25	36.4	45	44
		50	39.1	49	50
	Ex situ	25	32.4	40	42
		50	35.0	45	46
Tithonia	*In situ*	25	30.4	38	40
		50	34.7	42	42
	Ex situ	25	28.6	38	37
		50	31.2	40	41
None		25	23.5	34	31
		50	26.9	38	39
Probability					
Green manure			0.006	0.017	0.012
Addition			0.011	0.043	0.047
Fertilizer N			0.008	0.027	0.040
Green manure × addition			0.014	0.046	0.033
Green manure × N			0.003	0.011	0.042
Addition × N			0.008	0.043	0.019
Manure × addition × N			0.547	0.069	0.088

Table 2
Yield (kg ha^{-1}) and harvest indices of maize as affected by green manure application and nitrogen fertilizers (kg ha^{-1}) over major and minor seasons

Green manure	Addition	Fertilizer N	Seed yield	Harvest index
Major season				
Crotalaria	*In situ*	25	3842	0.42
		50	4245	0.41
	Ex situ	25	3514	0.37
		50	3941	0.39
Tithonia	*In situ*	25	3739	0.40
		50	4046	0.38
	Ex situ	25	3542	0.38
		50	3851	0.38
None		25	3637	0.39
		50		
Probability				
Green manure			0.024	0.142
Addition			0.005	0.487
Fertilizer N			0.041	0.662
Green manure × addition			0.007	0.524
Green manure × N			0.027	0.099
Addition × N			0.033	0.093
Manure × addition × N			0.067	0.541
Minor season				
Crotalaria	*In situ*	25	2829	0.39
		50	3134	0.38
	Ex situ	25	2541	0.37
		50	2714	0.38
Tithonia	*In situ*	25	2599	0.37
		50	2794	0.38
	Ex situ	25	2315	0.38
		50	2611	0.37
None		25	2014	0.37
		50	2255	0.36
Probability				
Green manure			0.005	0.604
Addition			0.037	0.551
Fertilizer N			0.040	0.821
Green manure × addition			0.009	0.743
Green manure × N			0.046	0.088
Addition × N			0.026	0.142
Manure × addition × N			0.156	0.214

A comparison of the impact of different methods of adding green manures shows the benefits of *in situ* application, and again the yield increments were greater in the minor season. The increments due to *in situ* green manuring with crotalaria were 8% and 13% in the major and minor seasons, while the increments due to *in situ* addition of tithonia were 5% and 8%, respectively, in these two seasons, which corresponds well to the vegetative growth. Thus, greater seed yields are clearly obtained with *in situ* green manuring, though again the reasons remain unclear.

As expected, the application of 50 kg N per ha increased seed yields compared with 25 kg N, and the increase in yields was similar in both seasons with both green manures. The increments mostly ranged from 8–10%, with the highest increment (13%) seen with the *ex situ* application of tithonia in the minor season. This suggests that green manures are important in providing a synergistic effect to applied fertilizers, and their application is thus an important practice in tropical smallholding systems in order to increase the yields of N-demanding species such as maize.

There were no significant differences in the harvest indices due to the different treatments adopted (Table 2) in either season. However, the lower harvest indices in the minor season illustrate poor yields irrespective of the treatments, confirming the benefits of a conducive climate for maize yields. The application of the green manures marginally increased the harvest indices in both seasons, especially when applied *in situ*, again indicating the benefits of growing a green manure before cropping when possible.

N harvest indices and N use efficiencies

The N harvest indices (NHI) and N use efficiencies (NUE) were significantly greater in the major season, irrespective of the treatments adopted, implying the better uptake and use efficiencies of applied N fertilizer in the more favourable season (Table 3). This could be associated with the lower loss of applied N from the soil in the major season, due to the better soil moisture conditions, which in turn enhances N availability to crops (Soon et al., 2008).

The application of green manures enhanced NHI and NUE significantly, as they are able to retain the applied N when compared to conventional farming without green manures (Fowler et al., 2004). The impact of the green manures on NHI was similar in both seasons when compared to the respective control treatments. However, the mean NUE values were increased to a greater extent in the major season, which again is due to the more conducive climate, where the greater uptake of applied N results in higher yields. The use of crotalaria increased both indices to a greater extent than tithonia in both seasons, due to the ability of the legume to complement the added fertilizer N, when compared to tithonia, which has a higher C:N ratio and may use some of the applied N for its decomposition. Furthermore, the *in situ* application of both green manures increased both NHI and NUE, a phenomenon similar to that of growth and seed yields, implying the benefits of this method of applying green manures.

The higher rate of N fertilizer also increased both NHI and NUE due to the greater availability of the nutrient in the soil for plant uptake, with no differences between the two green manures. *In situ* green manuring increased the NHI and NUE values significantly, again suggesting the benefits of this method of improving soils to enhance fertilizer use efficiencies. *Ex situ* green manuring, although effective in increasing both NHI and NUE, did not have the same impact on these parameters as *in situ* green manuring, as shown by the lower values.

Table 3
Impact of green manure addition and fertilizer N on N dynamics in maize grown in major and minor seasons

Green manure	Addition	Fertilizer N	NHI	NUE (%)*
Major season				
Crotalaria	*In situ*	25	68	32.5
		50	71	38.6
	Ex situ	25	65	30.4
		50	69	35.6
Tithonia	*In situ*	25	58	28.6
		50	64	30.5
	Ex situ	25	56	25.8
		50	61	28.9
None		25	31	15.6
		50	39	18.2
Probability				
Green manure			0.004	0.017
Addition			0.040	0.009
Fertilizer N			0.028	0.011
Green manure × addition			0.037	0.024
Green manure × N			0.029	0.008
Addition × N			0.031	0.039
Manure × addition × N			0.133	0.090
Minor season				
Crotalaria	*In situ*	25	58	22.5
		50	66	27.1
	Ex situ	25	52	20.4
		50	57	24.6
Tithonia	*In situ*	25	42	21.5
		50	48	24.2
	Ex situ	25	39	20.6
		50	42	22.5
None		25	21	12.4
		50	24	15.6
Probability				
Green manure			0.018	0.037
Addition			0.043	0.012
Fertilizer N			0.033	0.005
Green manure × addition			0.029	0.042
Green manure × N			0.017	0.036
Addition × N			0.008	0.004
Manure × addition × N			0.083	0.051

*NHI and NUE were calculated as presented in the text

Conclusions

Green manuring is considered a feasible and suitable method of increasing productivity and maintaining sustainability in tropical smallholding systems. However, these farming systems use various materials, which are applied *in situ*

or *ex situ*, with or without fertilizers. The field study carried out in Sri Lanka over the two tropical cropping seasons illustrated the benefits of green manuring to maintain yields in both the major and minor seasons. The impact is greater in the minor season due to the lower yields caused by the harsher climate, when the crops are subjected to moisture and even some heat stress. They have the ability to enhance vegetative growth and seed yields in the minor season. Even in the favourable major season, they have a significant impact on the growth and yield of maize. They also increase the utilization efficiency of applied N, the most common chemical fertilizer used in these regions, to a greater extent in the major season. A comparison of the two green manures highlighted the greater benefits of a legume species when compared to tithonia, which also has the potential of supplying P to crops (Jama et al., 2000). The green manures also increase the benefits of enhanced rates of N fertilizer to the crops. The comparison of the two methods of application clearly illustrated the benefits of *in situ* green manuring. Although this method ties up land for a short period of time between seasons, it has a greater beneficial effect, the cause of which could be the greater addition of organic material through leaf fall and roots, which were not quantified, and also the possibility that these roots improve soil properties, although these factors need confirmation. As most farmers in the tropics tend to add green manures from external sources, they need to be encouraged to grow these species *in situ*, as applying similar quantities from the hinterlands to crops does not provide the same benefits.

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LONG-TERM INFLUENCE OF ORGANIC AND INORGANIC FERTILIZERS ON NUTRIENT BUILD-UP AND THEIR RELATIONSHIP WITH MICROBIAL PROPERTIES UNDER A RICE-WHEAT CROPPING SEQUENCE IN AN ACID ALFISOL

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The long-term effect of organic and inorganic fertilizers on nutrient build-up and their relationship with microbial properties in a rice-wheat cropping sequence were studied in surface (0–15 cm) and subsurface (15–30 cm) soil samples. This experiment has been in progress since 1990 in the Department of Agronomy, CSK, Palampur with twelve treatments involving combinations of organic and inorganic fertilizers. In these treatment combinations, 25 and 50% of the recommended nitrogen levels were supplemented with organic sources, i.e. FYM (farm yard manure), green manure and wheat straw. The build-up of organic matter, and the total and available pools of nitrogen, phosphorus and sulphur were determined in surface (0–15 cm) and subsurface (15–30 cm) soil samples. The substitution of 25 and 50% nitrogen through organic fertilizers proved to be better than inorganic fertilizers alone. Amongst the organic sources, the substitution of FYM resulted in higher organic carbon, total and available nitrogen, phosphorus and sulphur than green manure or wheat straw. The substitution of 50% nitrogen through organic fertilizer was more effective than the substitution of 25% nitrogen. The relationships between the total and available pools of nutrients and the total microbial count, biomass carbon, microbial respiration, and the dehydrogenase and phosphatase enzymes were studied. The total pool of nutrients showed a high, positive, significant relationship with all these parameters.

Key words: Alfisol, biomass carbon, dehydrogenase, microbial counts, microbial respiration, phosphatase

Introduction

The need to produce food to nourish the burgeoning human population from a shrinking area of land with less water, has forced the application of more and more chemical fertilizers to achieve the desired quantity of food, which in turn has caused a decline in fertilizer use efficiency (FUE) and eroded the ecological environment. The reserve nutrients in the soil are not sufficient for the quantity of crop produce required for the increasing human population. So increases in food grain have been achieved at the cost of soil health and

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environment. It is now unanimously agreed that decreasing fertilizer use efficiency (FUE) and declining soil organic matter (SOM) levels are serious threats to sustainability.

Both the fertility and the health of the soil must be maintained to ensure sustained agricultural productivity. It is quite clear that no single source of plant nutrients, be it mineral fertilizers, organic manures, crop residues or bio-fertilizers, can meet the total nutrient requirements of the increased crop yields. Crops remove more nutrients every year than are supplied through the addition of chemical fertilizers, thus showing the contribution of organic and biological sources (Gaur, 1998). The combined use of organic manures and inorganic fertilizers influences the physical, chemical and biological properties of the soil and plays an important role in energy flow and nutrient cycling. It not only sustains higher levels of productivity, but also improves soil health and enhances nutrient use efficiency. Keeping this in view the present investigations had the following objectives: i) to examine the effect of organic and inorganic fertilizers on the total and available pool of nitrogen, phosphorus and sulphur at two soil depths; ii) to investigate the relationship between the total and available pools of nutrients and the microbial population, microbial biomass, and the carbon dehydrogenase and phosphatase enzymes at two soil depths.

Materials and methods

The long-term effect of organic and inorganic fertilizers has been studied in Palampur since 1990 in an experiment involving a randomized block design with twelve treatments and four replications.

The details of the treatments are as follows:

Treatments	Wet season (rice) (kg $N+P_2O_5+K_2O$/ha)	Cold season (wheat) (kg $N+P_2O_5+K_2O$/ha)
T_1	Control (no fertilizer, no manure)	Control (no fertilizer, no manure)
T_2	45+20+20	60+45+15
T_3	45+20+20	120+90+30 (recommended dose)
T_4	67.5+30+30	90+67.5+22.5
T_5	90+40+40 (recommended dose)	120+90+30
T_6	37.6 t FYM + 45+20+20	120+90+30
T_7	18.8 t FYM + 67.5+30+30	90+67.5+22.5
T_8	76.6 t WS + 45+20+20	120+90+30
T_9	37.6 t WS + 67.5+30+30	90+67.5+22.5
T_{10}	90.2 t Dh + 45+20+20	120+90+30
T_{11}	45.1 t Dh + 67.5+30+30	90+67.5+22.5
T_{12}	50 t FYM + 36+16+16 (Farmer's practice)	50 t FYM + 48+36+12 (Farmer's practice)

FYM: farmyard manure; WS: wheat straw; Dh: Dhaincha green manure

The experimental soil has been classified as a Typic Hapludalf belonging to the order Alfisol and developed from fluvioglacial parent materials. The soil texture was silty loam with the following characteristics: sand 22%, silt 54%, clay 24%; pH in a 1:2.5 (w/v) soil:water suspension 5.7, organic carbon 6.0 g kg^{-1}; available nitrogen 541 kg ha^{-1}; available phosphorus (P_2O_5) 29 kg ha^{-1} at the start of the experiment. Soil samples were collected from depths of 0–0.15 and 0.15–0.30 m after the harvest of wheat (2002–03). The samples were processed and stored in air-tight polythene bottles for subsequent microbiological and chemical analysis.

Microbiological studies

The microbial population (colony-forming units, cfu) was determined using the plate count technique of Wollum (1982) through serial dilution using a variety of media. The dehydrogenase activity was determined as described by Casida et al. (1964) and the phosphatase activity by the method of Tabatabai and Bremner (1969). The microbial biomass carbon was determined by the fumigation-extraction method of Vance et al. (1987) and microbial respiration as described by Stotzky (1965). The biomass specific respiration ratio was calculated using the formula:

Biomass specific respiration = Soil respiration/Microbial C

The relative pool of microbial carbon was calculated as:

Relative pool of microbial carbon = Microbial C/Soil organic C.

Chemical studies

Soil pH, organic carbon and total nitrogen, phosphorus and sulphur were determined by standard methods (Jackson, 1973).

Results and discussion

Organic carbon

It is clear from Table 1 that the organic carbon content differed significantly in the surface and subsurface soil in the different treatments. The continuous application of inorganic fertilizer alone reduced the organic carbon content from its initial status (6.0 g kg^{-1}). However, increasing NPK doses increased the organic carbon compared with the absolute control. This may be due to the better root biomass production and its subsequent decomposition, resulting in the increase in the organic carbon status of the soil (Chaudhary et al., 1981). The substitution of organic manure increased the organic carbon content from its initial status, higher rates contributing to the build-up of organic carbon in both the surface and subsurface soil. This increase may be due to the direct addition of carbon from organic sources along with the NPK fertilizers, which resulted in an enhanced rate of mineralization due to the narrowing of the C/N ratio. Similar results were reported by Tolanur and Bodanur (2003a, b) in the soils of Bihar. Amongst the treatments, the substitution of inorganic fertilizer by FYM (T_6 and T_7) gave a greater amount of organic carbon than green manure or wheat straw. Although wheat straw enhanced organic carbon as compared to inorganic fertilizer alone, it was less efficient than FYM or green manure due to the low decomposition of the more resistant form of organic matter. Farmer's practice (T_{12}) resulted in higher organic carbon than the absolute control or the use of inorganic fertilizer alone. In general, the organic C contents were higher at the surface than in the subsurface soil because of the accumulation of organic matter in the surface layer and the more efficient nutrient transformation in surface soils as the result of greater microbial activity.

Total and available nitrogen

The status of total and available nitrogen differed significantly in the different treatments (Table 1), being lowest in the control due to the drastic exhaustion of the native nutrient pool. Increased levels of NPK led to an increase in the total and available nitrogen, which could be attributed to better plant and root growth, whose subsequent decomposition increased the total and available nitrogen status of the soil as compared to the absolute control. Similar results were reported by Bharadwaj and Omanwar (1994) in the Tarai soils of Uttar Pradesh. The application of inorganic fertilizers did not cause a significant build-up of the available nitrogen status, though it proved to be better than the control. The substitution of organic sources resulted in significantly higher total and available nitrogen status than the absolute control, probably due to the residual effect of chemical degradation and mineralization. Among the organic sources applied, FYM gave the greatest total nitrogen, followed by wheat straw and green manure. The available nitrogen was the highest in the FYM treatments and the lowest in the wheat straw treatment at both depths. The results corroborated the findings of Sharma et al. (2000) in the soils of Jammu and Kashmir and of Singh et al. (2001) in the soils of Bihar.

Total and available phosphorus

The total and available phosphorus contents in surface (0–15 cm) and subsurface (15–30 cm) soils after the application of organic fertilizers and inorganic fertilizers are presented in Table 1, which shows that the total and available phosphorus differed significantly in the different treatments. The continuous application of 50% NPK (T_2 & T_3) resulted in a lower total phosphorus content in the surface soil than in the absolute control. However, the continuous application of 75 or 100% NPK gave higher total and available phosphorus in the surface and subsurface soil than in the absolute control. This may have been due to an increase in microbial activity, which in turn resulted in the greater production of carbon dioxide, which dissolves in water to form carbonic acid, which in turn dissolves primary minerals and releases the soluble fractions of phosphorus compounds (Bharadwaj and Omanwar, 1994). The maximum build-up of total phosphorus was obtained in the green manure treatment in both surface and subsurface soils, with the highest value in the continuous 100% NPK treatment. The combined application of organic and inorganic fertilizers increased the total and available phosphorus status, which could be attributed to the increase in organic forms of nutrients in the soil and to the enhanced activity of various microorganisms. The addition of organic manure provided a continuous source of carbon for the decomposition of organic matter and resulted in more humus, which might have facilitated the solubilization of native nutrients and protected them from further adsorption and precipitation (Das et al., 1991). Amongst the organic fertilizers, FYM gave the

best values of available phosphorus in both surface and subsurface soils, possibly because the narrow C:N:P ratio of FYM resulted in fast, continuous degradation. Farmer's practice (T_{12}) gave better P status than the absolute control, but could not compete with the recommended dose of NPK, as also reported by Tolanur and Bodanur (2003a, b) in the soils of Bihar.

Total and available sulphur

The data presented in Table 1 revealed that the total and available sulphur status differed significantly in the different treatments. Total and available sulphur was minimum in the absolute control and maximum when organic fertilizer was substituted. Among the inorganic fertilizer treatments, the application of 100% NPK consistently gave the highest total and available sulphur contents. This could be attributed to the fact that phosphorus was applied as single superphosphate, which contains 12% sulphur on an elemental basis, thus enhancing the total and available sulphur status of the soil (Tiwari et al., 1995). The data further revealed that the substitution of part of the nitrogen dose by organic manure led to higher values of sulphur than inorganics only. The increase in the sulphur status of the soil in the organic manure treatments stimulates the activity of microorganisms, while the application of single superphosphate helps to maintain a proper C:S ratio, which also stimulates microbial activity. So in the organic treatments, the soil obtained sulphur from both the organic and the inorganic nutrient pools (Stevenson, 1980). Amongst the organic resources, the highest total available S status at both soil depths was obtained in the FYM-treated plots, followed by green manure and wheat straw. FYM is a rich source of both micro- and macronutrients and has a balanced C:S ratio, which is essential for slow, continuous mineralization. These results corroborated the findings of Sharma et al. (2000) in the soil of Jammu and Kashmir. The plant used as green manure is a legume, so it has a high nitrogen concentration, resulting in the rapid decomposition of organic residue. Wheat straw, on the other hand, contains persistent carbonaceous material, resulting in poor, slow mineralization. As for the other nutrients, the total and available sulphur was higher in the surface soil than in the subsurface soil, because of the greater accumulation of organic matter on the surface (Sheeba and Chellamuthu, 1999).

Biomass specific respiration and relative pool of microbial carbon

The biomass specific respiration and the relative pool of microbial carbon were calculated and presented in Table 1, which shows that the biomass specific respiration increased with an increase in chemical fertilizers and after the combined application of organic and inorganic fertilizers. The maximum value was obtained in the T_8 treatment at both depths, because the microbial population was maximum in the wheat straw treatments. The relative carbon pool did not exhibit a consistent trend at either depth, but was maximum in T_7 in

the surface soil. The values of both these parameters tended to be higher in the surface soil than in the subsurface samples. Microbial activity was maximum in treatments T_6 to T_{11}, where organic and inorganic fertilizers were applied together. In the case of farmer's practice (T_{12}), though organic and inorganic fertilizers were both applied, the quantities were lower than in treatments T_6 to T_{11}.

Relationship between microbial properties and chemical properties

The relationship between the microbial parameters and the total pool of nutrients is presented in Table 2. Correlations were found between various microbial properties and soil properties. The microbial population was not correlated with the pH of the surface soil, but was positively and significantly correlated with that of the subsurface soil, as also reported by Stroo and Jencks (1982). The microbial population was positively and significantly correlated with organic carbon, and total nitrogen, phosphorus and sulphur in both the surface and subsurface soil. This might be due to the fact that the increases in total C, N, P and S maintained a proper C:N:P:S ratio for the mineralization process, resulting in an increase in the total microbial population. The microbial population also showed a positive correlation with microbial respiration, dehydrogenase and phosphatase activity, all of which are of microbial origin (Tabatabai, 1994), but it was not correlated with biomass carbon in the surface soil (0–15 cm), indicating that the fumigation extraction method is not suitable for acid soil, where the values of microbial biomass carbon are lower, as the food and energy (ATP) are derived from the same pool of organic matter (Vance et al., 1987). However, there was a significant correlation with biomass carbon in the subsurface soil (15–30 cm) owing to the smaller microbial population, which resulted in less competition for energy and food. The dehydrogenase enzyme showed a positive and significant correlation with organic carbon at both depths, but was only correlated with total N, P and S in the subsurface soil, which might be due to the fact that organic matter is the substrate for dehydrogenase activity and a proper supply of N, P and S is required for the maintenance of the C:N:P:S ratio for mineralization by microorganisms, since dehydrogenase originates from microbial synthesis. In the surface soil the dehydrogenase activity thus depends more on the organic carbon content than on the C:N:P:S ratio. Phosphatase activity was positively and significantly correlated with total phosphorus at both depths and with the soil pH, organic carbon and total sulphur in the subsurface soil. Biomass carbon showed a significant correlation with phosphorus in the surface soil and with total sulphur in the subsurface samples. It is reported that the concentration of phosphorus in microbial cells is higher than that of other nutrients and that microorganisms may cause the immobilization of phosphorus in the soil, if the concentration of soil phosphorus decreases more than that of microbial cells (Coyne, 1999). Therefore, the total phosphorus present in the soil showed a positive correlation with microbial biomass.

Table1

Effect of organic and inorganic fertilizers on the organic carbon, content of the soil and on the available and total pools of nitrogen, phosphorus and sulphur

Treatments	Organic carbon		Nitrogen				Phosphorus				Sulphur				BSR		RPMC	
			Available		Total		Available		Total		Available		Total					
	D_1*	D_2**	D_1	D_2	D_1	D_2	D_1	D_2	D_1	D_2	D_1	D_2	D_1	D_2	D_1	D_2	D_1	D_2
T_1	4.6	3.0	188.5	157.5	495.8	549.5	19.3	16.7	182.3	140.0	5.6	8.4	175.0	149.3	0.013	0.015	0.08	0.07
T_2	5.1	3.4	196.5	188.5	863.5	628.0	39.0	31.8	168.0	154.3	7.0	12.6	187.5	157.0	0.016	0.015	0.06	0.06
T_3	5.4	3.5	204.0	193.7	867.3	706.5	45.5	41.7	168.0	181.5	12.6	14.0	200.0	196.5	0.018	0.019	0.08	0.08
T_4	5.4	4.2	216.0	196.3	785.0	706.5	43.5	42.0	325.5	224.8	14.0	18.2	208.2	198.3	0.026	0.017	0.07	0.07
T_5	5.8	4.8	227.3	220.8	1099.5	785.0	59.5	53.7	309.0	267.0	14.6	18.6	225.0	208.0	0.024	0.023	0.08	0.05
T_6	7.6	5.5	270.3	238.8	1099.5	993.5	59.3	53.3	393.0	364.0	15.0	25.3	275.0	263.0	0.027	0.022	0.05	0.05
T_7	7.4	5.0	251.5	245.8	1020.8	785.0	55.3	49.0	365.0	329.0	15.4	21.9	262.5	241.0	0.024	0.024	0.14	0.05
T_8	7.2	4.7	231.3	203.8	1020.8	704.5	53.5	48.3	325.5	295.0	15.5	15.8	235.5	205.5	0.097	0.038	0.03	0.06
T_9	7.1	5.0	218.5	196.5	942.0	706.5	54.3	48.4	365.0	276.0	16.0	14.0	229.3	216.0	0.077	0.026	0.03	0.07
T_{10}	7.4	5.2	251.5	220.5	951.0	863.5	51.3	47.0	429.0	365.0	11.2	16.5	247.0	235.0	0.024	0.021	0.05	0.08
T_{11}	7.3	5.1	241.5	214.3	863.5	828.0	57.8	51.3	421.0	315.0	12.6	15.5	255.3	248.5	0.019	0.018	0.06	0.07
T_{12}	6.7	4.6	220.0	173.5	706.5	628.0	39.8	32.1	224.0	154.0	8.3	14.0	193.2	187.0	0.011	0.012	0.07	0.06
$SD_{0.05}$	1.19	1.0	56.03	60.38	40.86	58.00	3.29	3.67	34.04	21.29	6.14	12.37	14.68	16.47	–	–	–	–

*: D_1 = 0–15; **: D_2 = 15–30 cm; BSR: Biomass specific respiration; RPMC: Relative pool of microbial carbon

Table 2

Relationship between microbial population, microbial activity and its biomass carbon and the total and available pool of N, P and S nutrients

		OC	AN	TN	AP	TP	AS	TS	MR	DA	PA	MP	MBC
pH	D_1	0.138	0.152	0.018	0.073	0.058	0.0585	–0.036	–0.090	–0.067	0.065	0.005	0.161
	D_2	0.523**	0.530**	0.696**	0.017	0.436**	0.488**	0.562**	0.238	0.442**	0.441**	0.521**	0.133
OC	D_1		0.155	0.158	0.339*	0.421**	–0.145	0.578**	0.177	0.842**	0.080	0.348	–0.125
	D_2		0.131	0.283	0.048	0.359**	0.327*	0.456**	0.384**	0.679**	0.424**	0.536**	0.279
AN	D_1			–0.086	–0.086	0.027	0.259	0.043	0.053	–0.094	–0.010	–0.004	–0.088
	D_2			0.439**	0.025	0.127	0.218	0.169	0.053	0.077	0.219	0.122	–0.039
TN	D_1				0.321*	0.153	–0.184	0.253	–0.028	0.049	0.127	0.291*	0.244
	D_2				–0.065	0.428**	0.483**	0.438**	0.061	0.687**	0.273	0.403**	–0.096
AP	D_1					0.691**	–0.239	0.674**	–0.069	0.168	0.406**	0.529**	0.149
	D_2					–0.048	0.069	0.047	0.339*	–0.046	0.327*	0.162	0.029
TP	D_1						–0.108	0.692**	0.039	0.151	0.352*	0.609**	0.387**
	D_2						0.370**	0.657**	0.199	0.600**	0.554**	0.741**	0.527
AS	D_1							–0.196	0.126	–0.029	–0.032	–0.170	–0.026
	D_2							0.706**	0.084	0.281	0.440**	0.472**	0.029
TS	D_1								0.161	–0.0006	0.252	0.549**	–0.056
	D_2								0.248	0.418**	0.464**	0.667**	0.389**
MR	D_1									0.029	0.154	0.299*	–0.054
	D_2									0.218	0.345*	0.397**	0.191
DA	D_1										0.105	0.119	0.037
	D_2										0.411**	0.606**	–0.049
PA	D_1											0.403**	0.165
	D_2											0.619**	–0.065
MP	D_1												0.225
	D_2												0.369

OC: Organic carbon; AN: Available nitrogen; TN: Total nitrogen; AP: Available phosphorus; TP: Total phosphorus; AS: Available sulphur; TS: Total sulphur; MR: Microbial respiration; DA: Dehydrogenase activity; PA: Phosphatase activity; MP: Microbial population; MBC: Microbial biomass carbon; *,** Significant at the 5% and 1% level, respectively; D_1 = 0–15; D_2 =15–30 cm

Conclusions

The substitution of 50% nitrogen through organic fertilizer proved to result in a greater build-up of organic carbon, total and available nitrogen, phosphorus and sulphur than 25% substitution or fertilizer alone. FYM was found to be better than green manure and wheat straw for the maintenance of organic carbon, total and available nitrogen, phosphorus and sulphur. The total pool of nutrients was found to be a suitable parameter for studying the relationship with total microbial counts, biomass carbon, microbial respiration, and the activity of the dehydrogenase and phosphatase enzymes.

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EFFECT OF HEAVY METALS ON THE LEAF DISC FERRICYANIDE REDUCTION IN CUCUMBER

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The effect of heavy metals on the leaf plasma membrane electron transport systems was investigated in connection with the tissue Fe concentration in Fe-sufficient and Fe-deficient cucumber leaves. Ten M μPb in the nutrient solution inhibited leaf ferricyanide reduction by 20–26%, whereas 10 M μCd had a more drastic effect, with 80–83% inhibition. Ferricyanide reduction decreased by 14% when 1 mM Pb was applied *in situ* by vacuum infiltration into control leaf discs, whereas it decreased by 40% when 0.1 mM Cd was applied. Ferricyanide reduction was completely inhibited by 1 mM Cd. The ferricyanide reduction values were correlated with the heavy metal, Fe and chlorophyll concentrations in the leaves. A significant linear correlation was only found with the chlorophyll concentration. The data suggest that there are also direct effects on membrane-bound reductases, but these are of less significance. Using differentially Fe-deficient plants (grown with 0 to 300 nM Fe in the nutrient solution), a chlorophyll concentration of 0.9–1.0 mg g^{-1} fresh weight was estimated as the threshold for achieving the ferricyanide reduction levels found in the controls.

Key words: *Cucumis sativus,* chlorophyll, leaf disc, Pb toxicity, Cd toxicity, iron uptake, apoplasm, symplasm

Introduction

In recent decades, many studies have been devoted to the investigation of heavy metal toxicity in plants. The effect of Pb and Cd on growth, photosynthesis, water relations and transport processes has been described in numerous studies (for review see Fodor, 2002). One common feature of the toxic effects is the disturbance of membrane functions via various mechanisms. Free heavy metal ions may induce oxidative stress (Ruley et al., 2004; Geebelen et al., 2002; Rucińska et al., 1999; Skórzyńska-Polit et al., 2003; Romero-Puertas et al., 2002). Reactive oxygen species (ROS) may be formed and may cause

membrane damage, as shown for both Pb (Verma and Dubey, 2003) and Cd (Dixit et al., 2001). Cadmium was also reported to affect membrane potential and permeability (Llamas et al., 2000).

Both Pb and Cd influence membrane transport processes. ATP-ase activity was decreased by Cd (Fodor et al., 1995; Burzyński and Kolano, 2003), while the uptake of essential cations and anions was affected differently by Cd and Pb (Fodor, 2002). Cadmium may interact directly with Ca or Fe uptake. Patch-clamp studies on *Vicia faba* guard cell protoplasts showed that the Ca^{2+} channels were permeable to Cd^{2+} (Perfus-Barbeoch et al., 2002), while IRT1, a high-affinity Fe transporter, may also facilitate Cd transport (Cohen et al., 1998; 2004). Nutrient imbalances caused by heavy metal toxicity through a decrease in the performance of metabolic processes may influence electron transport processes indirectly or directly through the plasma membrane.

The cell membrane electron transport is involved in Fe uptake, nitrate reduction and also in enzymatic cycles connected to free radical scavenging. There are at least five different NAD(P)H-utilizing redox systems associated with the plasma membrane in plants, namely: (i) NADH oxidase I, (ii) Fe^{3+}-chelate reductase, (iii) NADH-cytochrome b_5 reductase, (iv) NAD(P)H-nitrate reductase and (v) NAD(P)H-quinone reductase (Lüthje et al., 1997). Most of the plasma membrane redox enzymes that have been purified and identified are capable of reducing ferricyanide, a non-permeating electron acceptor (Bérczi and Asard, 1995). Several investigations have assessed Fe reduction using ferricyanide (Moog and Brüggemann, 1994) in plants that follow Strategy I for Fe uptake (Marschner et al., 1986). These plants respond to Fe deficiency with induced 'turbo' electron transport (Bienfait, 1985). It has been demonstrated that the reduction of Fe (naturally available as Fe^{3+}-chelates) prior to its uptake is facilitated by a specific Fe^{3+}-chelate reductase (Robinson et al., 1999). The majority of the NADH-ferricyanide oxidoreductase activity in spinach leaf plasma membranes could be attributed to an enzyme similar to potato tuber NADH-cytochrome b_5 reductase (Askerlund et al., 1991), although this ferricyanide reductase was later identified as the NADH-monodehydroascorbate oxidoreductase enzyme (Bérczi and Møller, 1998). In contrast, more recent experiments with right-side-out plasma membrane vesicles isolated from maize roots showed a clear connection between NADPH oxidation and ferricyanide reduction (Menchkhoff and Lüthje, 2004). Moreover, studies on ferricyanide and ferric-chelate reduction (Asard and Bérczi, 1998; Lynnes et al., 1998) showed that although ferric-chelate reduction was always lower, the activities were comparable. In the light of these studies it can be deducted that although ferricyanide reduction by plant tissues may not be reliably identified on the basis of specific natural enzyme activity, the latter nevertheless provides useful information about the redox activity of plasma membranes and the *in vivo* supply of electron donors, indicative of the metabolic state of the plant.

Concerning the effect of heavy metals, Cd^{2+} was shown to increase the root ferric chelate reductase activity of Fe-sufficient sugar beet plants to 3.3–4.1-fold. Moreover, both Cd-EDTA and Pb-EDTA (though the latter only at 2 mM concentration) led to 2.3–3-fold greater reduction (Larbi et al., 2002; Chang et al., 2003). In Fe-deficient cucumber plants, Pb^{2+} did not affect the induction of root ferric-chelate reduction, whereas Cd^{2+} severely inhibited it (Alcántara et al., 1994). The reduction itself was not affected by Cd or Pb (except for Pb at concentrations as high as 2 mM) (Chang et al., 2003). When short-term (30–60 min) Cd and Pb treatment was applied to intact Fe-deficient sugar beet roots, both Cd and Pb decreased reductase activity, though the effect of free ionic Cd^{2+} was stronger than that of Cd-EDTA or Pb-EDTA (Chang et al., 2003). The NADH-ferricyanide oxidoreductase activity was decreased *in vitro* in isolated plasma membrane fractions by Cd and Pb in Fe-deficient cucumber seedlings, but this effect was not observed *in vivo* (Burzyński and Buczek, 1994). All the above experiments were made with root tissues.

In leaf plasma membranes the ferricyanide reducing activity was investigated on intact leaf segments (Dharmawardhane et al., 1987), isolated mesophyll cells (Neufeld and Brown, 1987), isolated protoplasts and cells (Macri et al., 1992) and purified leaf plasma membrane vesicles (Askerlund et al., 1991; Bérczi and Møller, 1998). The redox activity showed clear inducibility by light, thus connecting the process to photosynthesis. Ferric-chelate reduction by leaf tissues was lower than ferricyanide reduction, and was inducible by light but not by Fe deficiency (Brüggemann et al., 1993; de la Guardia and Alcántara, 1996; Larbi et al., 2001). The effect of heavy metals on the ferricyanide reduction or Fe-chelate reduction in the leaves has not yet been investigated. Therefore, the objective of the present study was to assess the effect of heavy metals on leaf plasma membrane electron transport systems in connection with the Fe concentration of the tissues.

Materials and methods

Plant material

Cucumber (*Cucumis sativus* L. cv. Joker) plants were grown in hydroponic culture on non-buffered, modified Hoagland solution of the following composition: 1.25 mM KNO_3; 1.25 mM $Ca(NO_3)_2$; 0.5 mM $MgSO_4$; 0.25 mM KH_2PO_4; 11.6 μM H_3BO_3; 4.5 μM $MnCl_2.4H_2O$; 0.19 μM $ZnSO_4.7H_2O$; 0.12 μM $Na_2MoO_4.2H_2O$; 0.08 μM $CuSO_4.8H_2O$. Iron was supplied as 10 μM Fe(III)-citrate. The Pb and Cd treatments began when the first leaf appeared (nine-day-old stage). Heavy metals were added to the nutrient solution as 10 μM $Pb(NO_3)_2$ or 10 μM $Cd(NO_3)_2$. Each plant was grown individually in a separate 400 ml pot in a growth chamber with 100–150 μmol s^{-1} m^{-2} PPFD at 70% relative humidity, 24°C and 14/10 h light/dark photoperiod. The solution was renewed every second day. Three- or four-week-old plants were used for the experiments.

In a separate experiment, Fe-deficient plants were grown in the same nutrient solution, except that Fe-citrate was supplied as 0, 10, 30, 100 and 300 nM and no heavy metals were added. The plants were harvested at the three-week-old stage of development.

Metal concentrations

Besides the total Fe, Pb and Cd concentrations, the non-apoplasmic (symplasmic + vacuolar) portion in the leaves was determined separately based on the method of Becker et al. (1992) and Zhang et al. (1995), as described in Fodor et al. (2005). Forty-eight disks (d=8 mm) were excised from the leaves with a cork borer. After weighing, the leaf discs were vacuum infiltrated (50 kPa) in 15 ml solutions containing 10 mM Na_2EDTA and 0.5 mM $CaSO_4$ (pH 4.1, unbuffered) for 30 min, after which the samples were shaken in the same solutions on a laboratory shaker for 2 h to reach an equilibrium between the solutions and tissues. (The vacuum was increased gradually and released once after 15 min.) After drying, the disks were weighed and digested in a pressure-controlled microwave oven with 65% HNO_3. Since this procedure removes most of the di- and trivalent cations from the cell walls and intercellular spaces, the metal concentrations measured in these washed leaf disks were considered as non-apoplasmic. The solution used for the infiltration of each sample was directly analysed for metal content, which was subsequently considered as apoplasmic.

As a control, another set of 48 disks were cut from each leaf level. After drying and weighing they were directly digested with 65% HNO_3. The data obtained from these samples were considered as the total concentration of the elements in the appropriate plant parts.

The elemental analyses of the digested samples were carried out with an EXTRA IIA total reflection X-ray fluorescence spectrometer. A Mo tube (50 kV, 38 mA) was used for the measurements. The integration time was 500 s. Ni was used as internal standard for the quantitative determination.

Ferricyanide reduction

A ferricyanide reduction assay was designed, partly based on the method of de la Guardia and Alcántara (1996). Thirty discs (5 mm diameter) were cut from the leaves with a cork borer, weighed and washed with 0.5 mM $CaSO_4$. The discs were then placed in 5 ml of the following assay solution: 2.5 mM KNO_3, 2.5 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM KH_2PO_4 and 0.5 mM $K_3Fe(CN)_6$ (pH 5.65, unbuffered). The solution was infiltrated into the tissues under a vacuum (50 kPa) generated by a vacuum pump for 30 min. The vacuum was increased gradually and released once after 15 min. The discs were illuminated with white light (180 $\mu mol\ s^{-1}\ m^{-2}$) during the infiltration. Then 4 ml aliquots were taken and added to a solution containing 2 mM bathophenantrolinedisulphonate (BPDS), 1 mM $FeCl_3$, 1.5 M Na-acetate and 0.1 M citric acid in 1 ml. Following the reaction described in Avron and Shavit (1963), the Fe reduction was determined as Fe(II)BPDS by measuring the absorbance at 535 nm with an extinction coefficient of 20.75 $\mu M^{-1}\ cm^{-1}$.

Non-enzymatic ferricyanide reduction was tested using the procedure described above, except that the $K_3Fe(CN)_6$ was omitted from the assay solution. After the 30 min infiltration the discs were removed from the solution and 0.5 mM $K_3Fe(CN)_6$ was added. After another 30 min reaction time, 4 ml samples were taken. Fe reduction was measured as described above. These measurements were made with discs from control and Cd-treated plants.

The effect of heavy metals applied *in situ* was investigated on control plants. The method was modified after Chang et al. (2003). In this case the assay solution described above was used with or without 10 μM, 100 μM and 1 mM $Pb(NO)_3$ or $Cd(NO)_3$, the sampling and measuring procedures being the same as those described above.

Chlorophyll concentration

Chlorophyll (chl) concentrations were determined spectrophotometrically in 80% acetone (containing 0.16% NH_3) extracts using the equations of Porra et al. (1989).

Statistical analysis

Each plant was grown in a separate container. In each experiment five to seven parallel treatments were applied. The data are presented as means ± standard error (SE), and one-way ANOVA followed by Tukey's test was performed to reveal significant changes between treatments using the Statistica 2000 software. For curve-fitting the Origin 5.0 software was used and the fitting method is indicated with the levels of significance on each figure. For non-linear curve fitting, the Chi-square test was applied to estimate significance levels.

Results

Effects of Cd and Pb on the growth and chl concentration of leaves

Four-week-old control plants developed eight leaves, while Pb-treated and Cd-treated ones developed only seven and four leaves, respectively, which is a good indicator of shoot growth inhibition by the two metals. There was no significant decrease in the leaf fresh weight (FW) of the Pb-treated plants, whereas in Cd-treated plants it decreased markedly (data not shown). Whilst in the leaves of Pb-treated plants slight chlorosis appeared only in the upper part of the shoot, plants exposed to Cd had chlorotic and partly necrotic leaves. The average chl concentration of the control leaves was 2500±125 μg/g FW, decreasing by 20% in Pb-treated plants, although the difference was not significant on every leaf storey. The chl concentration decreased by 70–90% in plants exposed to Cd.

The chl concentration of the second leaf in plants grown on heavy metal-free nutrient solutions containing Fe-citrate in a 0–300 nM concentration range increased with the Fe supplied following a saturation curve (Fig. 1).

Both the Pb and Cd concentration in the leaf tissues decreased from the oldest to the youngest leaves. The Pb concentration was lower than that of Cd in every leaf, being approximately 60% of the Cd concentration in the oldest one, but increasing to 80–90% of the Cd value in younger leaves (Table 1). The vacuum infiltration and washing procedure removed 48–56% of the Pb and 0–10% of the Cd from the tissues. The non-apoplasmic heavy metal concentration in the leaves also showed a decreasing tendency towards the youngest leaves, like the total concentration, but the difference between the Pb and Cd concentrations was larger (the Pb value was 35–45% that of Cd). This originated from a greater decrease in the non-apoplasmic Pb concentration as compared to Cd (Table 1).

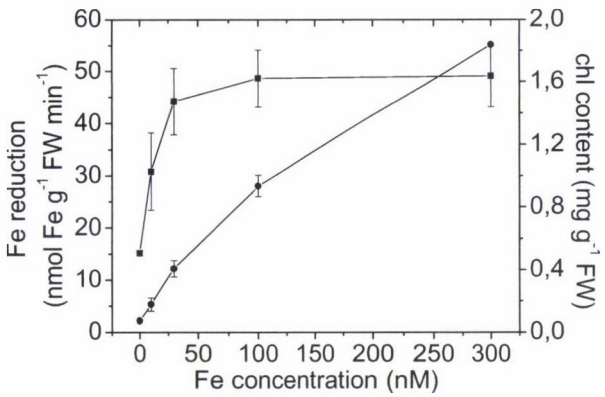

Fig. 1. Ferricyanide reduction (■) and chl concentration (•) in the second leaves of iron- deficient cucumbers grown for 3 weeks in nutrient solution as a function of the Fe-citrate supply. Data are presented as mean±SE, n=4

Table 1

Total and non-apoplasmic Cd and Pb concentration (μmol g^{-1} dry weight) in the leaves of 4-week-old cucumbers grown for 3 weeks in nutrient solutions containing heavy metal (10 μM Pb or Cd)

Leaf storey*	Pb total	Non-apoplasmic	Cd Total	Non-apoplasmic
1	3.85±0.15a	1.80±0.30bc	6.25±0.52f	5.60±0.47f
2	2.39±0.10b	1.19±0.05d	3.80±0.02a	3.46±0.02a
3	1.79±0.07bc	0.78±0.03d	2.05±0.06bc	1.86±0.06bc
4	1.44±0.03c	0.68±0.02de	1.86±0.04bc	1.86±0.04bc
5	0.96±0.02d	0.47±0.00e	–	–

*Leaves were numbered in the order of development; Data are presented as mean±SE, n=5. Values marked with different letters are significantly different (P<0.05, Tukey's test).

The iron concentration in the leaves was 3.2–5.8 μmol g^{-1} dry weight (DW) in control plants (Table 2). In Pb-treated plants it exceeded the control values or did not differ significantly. However, in Cd-treated plants the Fe concentration was very low (20–36% of the control). The non-apoplasmic Fe concentration in the leaves of the control was 2.7–4.0 μmol g^{-1} DW (Table 2). This was 68–87% of the total and increased towards the younger leaves. The non-apoplasmic Fe was between 30 and 60% of the total in Pb-treated leaves and 53–100% in Cd-treated leaves. In Pb-treated plants the non-apoplasmic Fe significantly decreased to 50–70% of the control values, while in Cd-treated plants it was fairly constant in all the leaves (23–25% of the control values).

The reducing capacity was investigated in leaf storeys two to five. The ferricyanide reduction in the leaves did not change significantly in relation with leaf age. The average value measured for the control leaves was 86.5±4.2 nmol Fe reduced g^{-1} FW min^{-1} (n=5). The ferricyanide reduction in Pb-treated plants was slightly below the control level (74–80%), except for leaf storey 5, which did not differ significantly from the control. The ferricyanide reduction in Cd-treated plants was 17–20% of the control.

Table 2

Total and non-apoplasmic Fe concentration (μmol g^{-1} dry weight) in the leaves of 4-week-old cucumbers grown for 3 weeks in nutrient solutions containing no heavy metal (control) or 10 μM Pb or Cd

Leaf storey	Control		Cd-treated		Pb-treated	
	Total	Non-apoplasmic	Total	Non-apoplasmic	Total	Non-apoplasmic
1	5.80±0.46a	4.02±0.32b	5.10±0.10a	2.20±0.07d	1.80±0.11d	1.01±0.06e
2	4.17±0.20b	3.34±0.16c	6.36±0.14a	1.92±0.04d	1.53±0.03d	0.85±0.02ef
3	3.59±0.18bc	2.97±0.15c	3.90±0.07b	2.05±0.04d	1.24±0.05e	0.66±0.03f
4	3.15±0.13c	2.69±0.11c	3.11±0.08c	1.86±0.05d	0.61±0.03f	0.61±0.03f
5	3.90±0.05b	3.41±0.04c	3.97±0.06b	2.17±0.03d	–	–

*Leaves were numbered in the order of development; Data are presented as mean±SE, n=5. Values marked with different letters are significantly different (P<0.05, Tukey's test).

In non-enzymatic reduction tests ferricyanide reduction activity was almost negligible in both control and Cd-treated plants, being approximately 5.0±0.9 nmol Fe reduced g^{-1} FW min^{-1} (n=3) in both cases, representing 5.8% of the control values in the direct enzymatic reduction measurements.

Both the total and non-apoplasmic heavy metal concentrations were compared with the ferricyanide reducing activity. There was no correlation between the corresponding data pairs in any of the leaves (plots not shown).

Effect of Pb and Cd applied in situ *on the ferricyanide reduction in the leaves*

The results showed no significant changes compared to the control value (72.0±3.1 nmol Fe reduced g^{-1} FW min^{-1} (n=5) when 10 or 100 μM Pb or 10 μM Cd was applied in the assay solutions. Ferricyanide reduction decreased by 14% when 1 mM Pb was applied, whereas it decreased by 40% for 100 μM Cd. Ferricyanide reduction was completely inhibited by 1 mM Cd.

Effect of different Fe concentrations on the ferricyanide reduction in the leaves

The rate of ferricyanide reduction in the yellow leaves of extremely Fe-deficient plants (grown without Fe) was 15.2±0.5 nmol Fe reduced g^{-1} FW min^{-1} (Fig. 1). This value is approximately18% of the Fe-sufficient control and in the same range as the Cd-treated values. The ferricyanide reducing activity increased following a typical saturation curve as the Fe-citrate concentration in the nutrient solution increased, but only reached 57% of the average value in Fe-sufficient control leaves at 300 nM Fe.

The ferricyanide reductase activity of plants treated with heavy metal and grown on 10 μM Fe-citrate was tested for a possible relationship with the Fe concentration. The higher the non-apoplasmic Fe concentration the higher the ferricyanide reduction. However, the non-linear curve fit was not statistically significant for either the total (not shown) or the non-apoplasmic Fe concentration in either Pb- or Cd-treated plants or in the control (Fig. 2), possibly due to the discontinuous data set.

When comparing the leaf chl with the non-apoplasmic Fe concentration of plants treated with heavy metal, a significant correlation was found, exhibiting a saturation curve (Fig. 3). However, the ferricyanide reduction by the leaves of these plants failed to show saturation kinetics with chl concentration; instead, a highly significant linear correlation was found (control, +Pb, +Cd, $P<0.0001$, Fig. 4).

Plants grown under Fe deficiency in a broad Fe concentration range (up to 300 nM) showed a good correlation, exhibiting a hyperbola type curve, between leaf chl concentration and ferricyanide reducing activity (Fig. 4). When all the data obtained for plants treated with heavy metal were inserted into the diagram, the chl – ferricyanide reduction values of the Pb-treated plants gave a good match to the 'saturated' section, while those of the Cd-treated plants were close to the initial section of the curve. When clustering all the data together in Fig. 4, a significant linear correlation was obtained ($P<0.0001$).

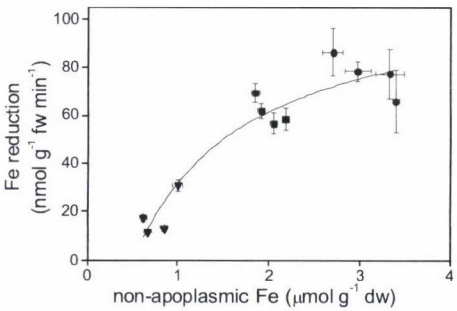

Fig. 2. Ferricyanide reduction in the leaves of 4-week-old cucumbers grown with no heavy metal (control) (•) or in nutrient solutions containing 10 μM Pb (■) or Cd (▼) for 18–19 days as a function of the non-apoplasmic Fe concentration. Data are presented as mean±SE, n=5. Non-linear curve fit: $y=a-(b/(1+cx)^{1/d}$, $Chi^2=85.08$

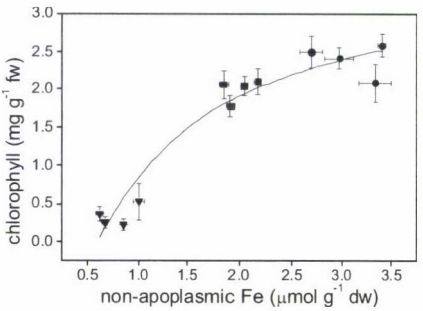

Fig. 3. Chl concentration in the leaves of 4-week-old cucumbers grown with no heavy metal (control) (•) or in nutrient solutions containing 10 μM Pb (■) or Cd (▼) for 18–19 days as a function of their non-apoplasmic iron concentration. (2^{nd}–5^{th} leaves were used for Control and +Pb and 1^{st}–4^{th} leaves for +Cd.) Data are presented as mean±SE, n=5. Non-linear curve fit: $y=a-(b/(1+cx)^{1/d}$, $Chi^2=0.06$

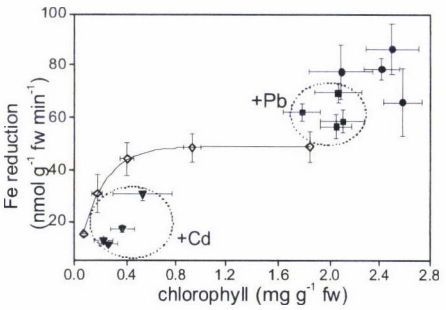

Fig. 4. Ferricyanide reduction in the leaves of cucumbers given various treatments, as a function of their chl concentration. –Fe (◊): 2^{nd} leaves of iron-deficient plants grown for 3 weeks (mean±SE, n=4), non-linear curve fit: $y=a(1-e^{-bx})$, a=49.15±0.42, b=5.48±0.17, $Chi^2=0.38$; control (•), +Pb (■): 2^{nd}–5^{th} leaves and +Cd (▼): 1^{st}–4^{th} leaves of iron-sufficient plants grown for 4 weeks (mean±SE, n=5), linear fit: R=0.98, SD=1.89, n=12, P<0.0001; linear fit for all data: R=0.92, SD=9.97, n=17, P<0.0001

Discussion

It is well established that Pb has a much weaker effect than Cd, if any, on the root cell membrane electron transport. The present data show that the ferricyanide reducing activity of the leaf tissues was inhibited when 10 μM Pb or Cd was applied in nutrient solution containing Fe-citrate and that Cd had a much stronger effect. This result is consistent with the fact that the concentration of Cd in the leaves was much higher than that of Pb. However, toxic heavy metals reach the leaf at much lower concentrations than those found in root tissues (Varga et al., 2002) and this depends on leaf age (Table 1). Though metals accumulate in the apoplasm, where they may exert an effect on the membranes, their uptake into the cytosol may facilitate a more direct effect on enzymes and the metabolism. Therefore, an attempt was made to determine the amount of Pb and Cd associated with the non-apoplasmic fraction of the leaves. The procedure used – vacuum infiltration of EDTA into leaf discs – has been applied in other studies for different purposes (Becker et al., 1992; Zhang et al., 1995) and is expected to scavenge EDTA-extractable metals from the apoplasm. EDTA was reported to cause membrane leakage (Vassil et al., 1998), though the diffusion to and from the leaf tissues is rather slow compared to the roots, so the results should be treated with caution.

EDTA removed Pb from the tissues more efficiently than Cd (Table 1). This could be explained by the higher stability constant of the Pb-EDTA complex (logK=18.0) versus that of the Cd-EDTA complex (logK=16.5). Since the high EDTA concentration employed (10 mM) ensures that all di- and trivalent metals are mobilized, the results cannot be explained solely via a stability constant comparison of Cd and Pb EDTA complexes. The diffusional properties of the various EDTA-metal complexes are also different, but the 2 h diffusion time was probably enough to reach equilibrium in both cases. It can thus be accepted that a larger portion of the Cd was located inside the cells as compared to Pb, which is in agreement with previous findings showing that most of the accumulated Pb remains in the apoplasm (Varga et al., 2002) and that Cd can be taken up by the cell through IRT1, the divalent Fe transporter (Cohen et al., 1998; Conolly et al., 2002) or Ca channels (Perfus-Barbeoch et al., 2002).

Neither the total nor the non-apoplasmic metal concentration determined in this way could account for the decrease in ferricyanide reducing activity, i.e. no significant correlation was found between the corresponding data pairs in each leaf.

The *in situ* effect of the metals investigated by the vacuum infiltration of control leaves was different from the *in vivo* effect. None of the metals decreased the reducing activity at the lowest concentration used. Higher concentrations, however, resulted in increased inhibition, though the specificity of the effect is questionable. As the root plasma membrane NADH-ferricyanide-oxidoreductase showed similar behaviour *in vivo* and *in vitro* (Burzyński and

Buczek, 1994) the conclusion reached is that the heavy metals applied have no direct effect on the leaf plasma membrane electron transport system or that inhibition is triggered by large concentrations that may never occur *in vivo*. However, the inhibition may also come from the interference of Cd with SH-groups (van Assche and Clijsters, 1990), which would be implied by the great sensitivity of the leaf plasma membrane Fe-chelate reductase to *p*-hydroxy-mercuribenzoic acid (*p*HMB), a specific –SH group reagent (de la Guardia and Alcántara, 1996).

In order to evaluate the indirect effects of heavy metals on the ferricyanide reduction, other physiological parameters were also examined. Significant growth inhibition was only observed in Cd-treated plants, which were also severely chlorotic. This is in agreement with other studies (Burzyński and Buczek, 1994; Titov et al., 1996; Sanità di Toppi and Gabbrielli, 1999). The chl concentration of Pb-treated plants also decreased, in agreement with previous findings demonstrating the inhibition of δ-aminolevulinic acid synthesis by Pb (Sengar and Pandey, 1996). Consequently, it seems clear that the chl synthesis must have been affected in both cases. Similar findings have been reported by several authors (e.g. Gadallah, 1995; Geebelen et al., 2002; Hsu and Kao, 2003). It is also known that Cd severely inhibits the uptake of Fe, which is essential for chl synthesis, and its transport to the shoot (Fodor et al., 1996; Yang et al., 1996; Zhang et al., 2000). In the present study Fe accumulation was not modified or even stimulated by Pb, whereas it was markedly decreased by Cd (Table 2). When the total Fe concentration values were compared with the ferricyanide reducing activity there was no correlation. Fe inside the cells showed a weak correlation, exhibiting a saturation curve and suggesting that an increase in cytoplasmic Fe concentration might facilitate reduction (Fig. 2). These results are in agreement with other studies showing that the total Fe concentration in leaves treated with heavy metal does not significantly correlate with the decrease in various physiological parameters, suggesting a smaller, active Fe pool (Láng et al., 1998; Sárvári et al., 1999).

As the chl concentration is an important parameter of the photosynthetic apparatus and an indicator of healthy Fe supply to the leaf cells, it was also compared with the ferricyanide reduction of leaves treated with heavy metal. However, a weak correlation of the saturation type was found, similar to that between the symplasmic Fe and chl concentrations (Fig. 3). These findings suggest that the heavy metals examined may exert their effect not only by decreasing the chl concentration, in connection with the inhibition of Fe uptake, but also by affecting the leaf plasma membrane electron transport system in some other way, e.g. by interfering with or down-regulating the expression of proteins not affected during short *in situ* treatment.

It is known that, in the absence of heavy metal treatment, there is a close positive correlation between total leaf Fe and chl concentration when the amount of Fe chelates supplied in nutrient solution is kept at suboptimal levels (Römheld

and Marschner, 1981; Terry and Abadía, 1986). In order to separate the effect of heavy metals from that of low chl concentrations, differentially chlorotic plants were grown by applying different Fe concentrations (between 0 and 300 nM) in the growing medium. The resulting, increasingly chlorotic plants were tested for leaf ferricyanide reduction (Fig. 1). The values exhibited a saturation curve with a steeply increasing section at low Fe concentrations, clearly showing that leaf ferricyanide reduction activity is not inducible by Fe deficiency but, on the contrary, requires Fe. The same was demonstrated for the electron transport activity connected to leaf Fe uptake (i.e. ferric chelate reductase activity) (de la Guardia and Alcántara, 1996), while in the roots both ferric chelate reductase and ferricyanide reductase activities are inducible.

The ferricyanide reduction rate in Cd-treated plants was at the Fe-deficient level, implying that Cd might influence the rate via interference with iron uptake, which is reflected in the chl concentration in the leaves. Using data pairs from the same leaves the leaf ferricyanide reduction was plotted as a function of chl concentration and a saturation curve was obtained, suggesting that from 0.9 to 1.0 mg chl g^{-1} FW there is a threshold level for the synthesis of the reducing equivalents necessary for the ferricyanide reduction (Fig. 1 and Fig. 4, '–Fe'). In leaf tissues, NAD(P)H generation is related to the photosynthetic activity, which strongly depends on the chl concentration (de la Guardia and Alcántara, 1996). Data pairs for ferricyanide reduction and chl concentration in the leaves of both Cd- and Pb-treated plants and the untreated control do not give a significant curve-fit with equations describing saturation, but give a highly significant linear fit. There is an obvious linear correlation between chl concentration and ferricyanide reduction, especially if all the data plotted in Fig. 4 are considered. This can be explained by the fact that Cd-treated plants are so chlorotic that they fit the Fe-deficient region of the curve, while Pb-treated plants fit the control level. Taking into account the low cytoplasmic concentration of heavy metals – especially of Pb – and the possible phytochelatin action and consequent sequestration in the vacuole (Rauser, 1995), this finding might also indicate a direct effect, e.g. the suppression of –SH inhibition by Fe deficiency chlorosis (confirmed by the '*in situ*' experiment or the low reduction rate of Cd-treated plants with a higher chl concentration than the Fe-deficient plants) or other side-effects of heavy metals, especially in the case of Cd. In conclusion, the decrease in leaf plasma membrane electron transport activity in Cd- and Pb-treated plants can be explained by an effect on the photosynthetic apparatus chiefly via the inhibition of chl synthesis or the inducing of chl degradation, while the direct *in vivo* inhibition of oxidoreductase enzymes located in leaf plasma membranes is possibly less significant.

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ROLE OF SALICYLIC ACID IN REGULATION OF CADMIUM TOXICITY IN WHEAT (*Triticum aestivum* L.)

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Treatment with $CdCl_2$ (0, 100, 400 and 1000 μM) resulted in the inhibition of root dry biomass and root elongation and to increased Cd accumulation in the roots. These treatments also decreased the relative water content, chlorophyll content, ^{14}CO fixation, phosphoenol pyruvate carboxylase and ribulose-1,5-bisphosphate carboxylase activity and abscisic acid (ABA) content, while increasing the malondialdehyde, hydrogen peroxide and free proline contents and causing changes in the chloroplast and root ultrastructure. Pretreatment of seeds with SA (500 μM) for 20 h resulted in the amelioration of these effects.

Key words: *Triticum aestivum* L., photosynthetic activity, chloroplast

Abbreviations: MDA – malondialdehyde; PEPC – phosphoenol pyruvate carboxylase; RuBPC – ribulose 1,5-bisphosphate carboxylase; RWC – relative water content; SA – salicylic acid; Cd – cadmium.

Introduction

Wheat (*Triticum aestivum* L.) is the most widely grown crop in the world, though best adapted to the temperate zone, and is the staple food of about 35% of the world population. Wheat is considered to be the major strategic food crop in Egypt. In addition, wheat straw is an important fodder (Gomma, 1999). The intensive use of high-phosphate fertilizers in agriculture has led to the increased accumulation of metal ions, especially cadmium, in the soil (Taylor, 1997). Cadmium is one of the most important metals in terms of food-chain contamination, because it is readily taken up by the cells of various plant species (Liu et al., 2007). In fact, at least 70% of the Cd intake by humans originates from plant foods (Wagner, 1993), leading to a potential threat to human health (Shah and Dubey, 1998). Cadmium has been shown to cause many morphological, physiological, biochemical and structural changes in plants, such

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as water imbalance, inhibition of seed germination and photosynthesis, reduction of growth, especially of root growth, disturbances in mineral nutrition and sugar metabolism, and therefore strongly influences biomass production (Sanità di Toppi and Gabbrielli, 1999; Moussa, 2004), finally leading to the death of the plant (Kahle, 1993). Cadmium produces alterations in the functionality of membranes by inducing changes in lipid composition (Ouariti et al., 1997), affecting the enzymatic activities associated with membranes, such as that of H^+-ATPase (Fodor et al., 1995), and decreasing the photosynthetic rate due to the reduced chlorophyll content and the enzymatic activity involved in CO_2 fixation (Greger and Ögren, 1991). In many plants Cd enhances the level of lipid peroxidation and causes alterations in antioxidant systems (Somashekaraiah et al., 1992). The harmful effects of Cd^{2+} might be explained by its ability to inactivate enzymes, possibly through reaction with the SH-groups of proteins (Gouia et al., 2004). Consequently, the alleviation of environment pollution by Cd is a growing concern in the research community.

Salicylic acid is a phenolic compound commonly occurring in vascular plants. It plays an essential role in the regulation of plant growth and development and in plant responses to environmental stress (Senaratana et al., 2000). The exogenous application of SA increased yield (Raskin, 1992). Further, SA retarded ethylene synthesis, affected membrane depolarization, stimulated the photosynthetic apparatus and protein synthesis, and increased the content of chlorophyll (Khan et al., 2003; Shakirova et al., 2003). Salicylic acid mediates some positive acclimation responses to abiotic stress factors, such as heavy metals, herbicides, low temperatures and salinity (Janda et al., 1999; Metwally et al., 2003). SA pretreatment alleviates Cd toxicity in barley (Metwally et al., 2003) and maize plants (Krantev et al., 2008). It has recently been found that SA treatment caused both ABA and proline accumulation in wheat and increased resistance to salinity (Shakirova et al., 2003). SA ameliorates the damaging effects of heavy metals, such as lead and mercury (Mishra and Choudhuri, 1999).

The present study investigated the possible mediatory role of salicylic acid in alleviating cadmium toxicity. Morphological, physiological, biochemical and anatomical changes in parameters associated with oxidative stress, namely root length, root dry weight, Cd content, relative water content (RWC), free proline level, ABA production, lipid peroxidation, H_2O_2 content, chlorophyll content, photosynthetic efficiency ($^{14}CO_2$ fixation) and the activity of the carboxylating enzymes (RuBPC and PEPC), were assessed since they are known to be most affected by Cd treatment. Also, the ultrastructure of the root cells was assessed, as knowledge of the subcellular localization and identification of cadmium may provide essential information on metal toxicity and bioaccumulation mechanisms.

Materials and methods

Plant materials and growth conditions

A homogeneous lot of wheat seeds (*Triticum aestivum* L.) cv. Giza 155 was obtained from the Crop Institute, Agricultural Research Center, Giza, Egypt and stored at 4°C. The seeds were surface sterilized in 0.1% (w/v) sodium dodecyl sulphate solution, thoroughly rinsed with sterile deionized water and then presoaked for 20 h, either in 500 μM SA or in distilled H_2O as a control. SA was initially dissolved in a few drops of dimethylsulphoxide and the final volume was reached using distilled water. The seeds were then germinated in the dark at 24°C for five days. Selected healthy seedlings of equal size and vigour were transplanted to 30 cm black polyethylene pots containing 3 L of continuously aerated full-strength Hoagland's nutrient solution (Rafi and Epstein, 1999). Various cadmium concentrations (0, 100, 400 and 1000 μM) were added to the nutrient medium to give eight experimental groups: 1) control plants on nutrient solution without Cd^{2+}; 2) presoaked in water and treated with 100 μM Cd^{2+}; 3) presoaked in water and treated with 400 μM Cd^{2+}; 4) presoaked in water and treated with 1000 μM Cd^{2+}; 5) presoaked in SA without Cd^{2+}; 6) presoaked in SA and treated with 100 μM Cd^{2+}; 7) presoaked in SA and treated with 400 μM Cd^{2+}; 8) presoaked in SA and treated with 1000 μM Cd^{2+}. Cd was provided as cadmium chloride ($CdCl_2.2.5H_2O$). Double-distilled water was used for the nutrient solution and Cd solutions. The plants were grown in a controlled growth chamber under the following growth conditions: 15-h photoperiod; 65–75% relative humidity; day and night temperature of 22°C and 20°C. The photosynthetic photon flux density at maximum plant height was about 440 μmol $m^{-2}s^{-1}$. Five seedlings were planted in each pot. Each treatment was replicated four times and each replicate consisted of four pots. All the plants were harvested one month after treatment and separated into leaves, stem and root. The leaves were stored at –20°C prior to proline determination.

Growth and Cd accumulation

The growth of the root was studied in terms of root length (cm) and dry biomass, recorded after drying at 70°C for 2 days. The dried roots were digested in glass tubes containing 5 ml concentrated nitric acid at 100°C until the solution turned clear. The final volume was adjusted to 20 cm^3 with distilled water. Total Cd content was measured using an Atomic Absorption Spectrometer (Perkin-Elmer, 3110).

Determination of relative water content

The level of water deficit in the leaves was estimated on the basis of the relative water content determined by the method of Bandurska (1991).

Photosynthetic activity ($^{14}CO_2$ fixation)

Photosynthetic activity ($^{14}CO_2$ assimilation) was measured in the Atomic Energy Authority Radioisotope Department, Cairo, Egypt, with the method reported by Moussa and Abdel-Aziz (2008). One pot from each treatment was placed under a Bell jar, which was used as a photosynthetic chamber. Radioactive $^{14}CO_2$ was generated inside the chamber by a reaction between 10% HCl and 50 μCi (1.87×10^6 Bq) $NaH^{14}CO_3$ + 100 mg Na_2CO_3 as a carrier. The samples were then illuminated with a tungsten lamp. After 30 min exposure time, the leaves were quickly detached from the stem, weighed and frozen for 5 min to stop the biochemical reactions, then subjected to extraction by hot 80% ethanol. The ^{14}C in the soluble compounds was assayed from the ethanolic extracts using a Bray cocktail (Bray, 1960) and a Liquid Scintillation Counter (LSC2-Scaler Ratemeter SR7, Nuclear Enterprises).

Chemical analysis

The chlorophyll content was quantified according to Porra et al. (1989). Free proline was determined according to the method described by Bates et al. (1973). The lipid peroxidation level was measured biochemically in terms of malondialdehyde, a peroxidation product of fatty acid from membrane lipids, using the thiobarbituric acid reaction as described by Madhava Rao and Sresty (2000). The contents of hydrogen peroxide were measured according to Patterson et al. (1994). The isolation and estimation of ABA were performed by high-performance liquid chromatography (HPLC) according to Bandurska and Stroinski (2003).

Enzyme assay

The activity of phosphoenol pyruvate carboxylase was determined as described by Blanke et al. (1986). Ribulose-1,5-bisphosphate carboxylase was determined by Warren et al. (2000).

Transmission electron microscopy investigation

The roots were harvested after 15 days of culture. The terminal 2–3 mm section from each root and small segments of leaf (70–90 nm in thickness) from control and treated groups were cut and fixed in 2×5% glutaraldehyde in phosphate buffer (pH 7.2) for 4 h and then thoroughly washed with the same buffer three times. This was followed by post-fixation with 2% osmium tetroxide in the same buffer for 2 h. The samples were dehydrated in an acetone series and embedded in ERL resin. For ultrastructure observations, ultra-thin (80 nm) sections were cut on an ultramicrotome (Ultracut E, Leica, Germany) with a diamond knife, floated on water and collected on copper grids. The sections were stained with 1% uranyl acetate for 1 h and with lead citrate for 15 min. They were examined and photographed using a transmission electron microscope (TEM, Jeol Jem 1200 EX II, Tokyo, Japan).

Statistical analysis

All the experiments were repeated at least four times and the data presented are the means of four separate experiments±SE. The statistical significance between the treatments was given by the *t*-test.

Results

A significant decline in root length and dry weight was observed in treatments without SA. SA priming resulted in an initial increase in root length and dry weight (Table 1), with a minor reduction at 1000 μM of Cd. In the controls, no Cd was detected. Cd accumulation was significantly increased in SA-free roots as compared to those primed with SA (Table 1). Exposure of wheat plants to Cd^{2+} led to a slight decrease in leaf RWC. SA priming resulted in a significant increase in RWC as compared to the SA-free controls (Table 1). The growth inhibition of wheat plants was accompanied by a significant decrease in the chlorophyll content, the rate of photosynthesis (14CO2 assimilation) and the activities of carboxylating enzymes (RuBPC and PEPC). Pretreatment of wheat plants with SA before exposure to Cd alleviated the inhibitory effect of Cd on these parameters (Table 2). The stress metabolites, H_2O_2, MDA and free proline, increased significantly with increasing Cd concentrations, the greatest effect being observed at 1000 μM Cd (nearly three-fold rise in free proline content compared to the control). SA pretreatment alleviated the harmful effect of Cd and significantly decreased the level of these parameters (Table 3).

Table 1
Growth parameters and root Cd content (mg g^{-1} DW) in wheat plants under Cd toxicity and SA free/primed conditions

Treatment	Root length (cm)	Root dry mass (g)	Root Cd content	RWC (%)
Control	12.9±0.38	0.23±0.002	0.0±0.0	94.7±5.7
100 µM Cd	11.2±0.22*	0.20±0.010	3.9±0.234*	91.3±7.3
400 µM Cd	9.4±0.41**	0.17±0.007*	6.8±0.544*	87.1±2.6*
1000 µM Cd	7.2±0.36***	0.10±0.006*	17.5±1.963***	75.8±3.8**
Control+SA	15.9±0.85	0.32±0.028	0.0±0.0	96.1±6.7
100 µM Cd +SA	13.6±0.95*	0.27±0.011*	1.8±0.126*	94.2±10.3
400 µM Cd +SA	11.9±0.47*	0.22±0.009	4.3±0.602**	90.6±4.5*
1000 µM Cd +SA	9.9±0.29***	0.16±0.008*	7.4±0.731**	83.7±3.1**

Data are the means of four separate experiments±SE. *, **, ***: Differences significant at the $p<0.1$, $p<0.01$ and $p<0.001$ levels, respectively

Table 2
Chlorophyll content (µg g^{-1} FW), $^{14}CO_2$ fixation (kBq mg^{-1} FW) and carboxylating enzyme activity (µmol NADH mg $protein^{-1}$ min^{-1}; µmol RuBPC mg^{-1} min^{-1}) in wheat plants treated with Cd or pretreated with SA before exposure to Cd

Treatment	Chlorophyll a+b	Photosynth. act.	PEPC activity	RuBPC activity
Control	3.74±0.18	18673±933	25±1.7	78±4.7
100 µM Cd	3.42±0.20	17125±685	21±1.6	73±2.9
400 µM Cd	3.00±0.21*	12378±1237**	13±0.9*	52±3.1*
1000 µM Cd	1.78±0.23**	5986±239***	6±0.2**	28±2.8***
Control+SA	3.86±0.23	20721±1243	27±2.3	82±5.2
100 µM Cd +SA	3.68±0.14	19466±1946	25±1.5	79±4.7
400 µM Cd +SA	3.50±0.31*	16611±1328**	19±0.5**	67±5.4**
1000 µM Cd + SA	2.56±0.28**	10984±1318**	14±0.7***	46±1.8***

Data are the means of four separate experiments±SE. *, **, ***: Differences significant at the $p<0.1$, $p<0.01$ and $p<0.001$ levels, respectively

Table 3
Contents of MDA (µmol g^{-1} FW), H_2O_2 (µM g^{-1} FW), free proline (nM g^{-1} FW) and ABA (nM g^{-1} FW) in SA-free and SA-primed wheat plants under Cd toxicity

Treatment	MDA	H_2O_2	Free proline	ABA
Control	388±23.3	99±5.9	250±15	0.695±0.061
100 µM Cd	413±33.1	138±5.5*	384±46*	0.718±0.072
400 µM Cd	497±44.7*	167±11.7**	508±43**	0.501±0.031*
1000 µM Cd	563±56.3**	183±12.5**	722±52***	0.208±0.023***
Control+SA	281±16.9	84±1.7	166±14	0.994±0.051
100 µM Cd +SA	319±19.1*	93±8.4	196±8*	1.035±0.046
400 µM Cd +SA	290±14.3**	110±6.6*	216±18**	0.876±0.062**
1000 µM Cd +SA	185±7.4***	125±8.7**	274±25**	0.549±0.084***

Data are the means of four separate experiments±SE. *, **, ***: Differences significant at the $p<0.1$, $p<0.01$ and $p<0.001$ levels, respectively

The ABA content decreased significantly with increasing Cd concentrations. SA priming resulted in a significant increase in ABA content as compared to the SA-free control (Table 3). Root-tip cells of control wheat plants had a typical ultrastructure, with highly condensed cytoplasm containing numerous organelles and a large nucleus. Ribosomes were richly distributed in the cytoplasm or located on the surface of the endoplasmic reticulum (Fig. 1a). Small vacuoles were found in the meristematic cells, whereas large vacuoles or several vacuoles were exhibited in mature parenchyma cells in the root-tips of plants treated with SA before exposure to Cd (Fig. 1b). Root cells exposed to various concentrations of Cd exhibited ultrastructural changes compared to SA-free root cells (Fig. 1a) or control root cells primed with SA (Fig. 1b). At 100 μM Cd, advanced vacuolation was the main toxic symptom observed in the meristem and cortical cells of the root-tips (Fig. 1c). This was more pronounced in cortical parenchyma cells than in stellar parenchyma cells in mature root tissues. From concentrations 400 to 1000 μM Cd, the structures of cytoplasm and organelles, both in cortex and stellar parenchyma tissues, were damaged in varying degrees. A reduction was observed in the number of mitochondria (Fig. 1d). The multivesiculate bodies were more pronounced in the parenchyma cells (400 μM Cd). Nucleus disintegration was accompanied by nucleoli containing rich electron-dense granules (Fig. 1e). Damaged membrane systems and serious plasmolysis with separations of the plasma membrane from the cell wall were noted in most cells in mature root tissues exposed to 1000 μM Cd. Structural damage to the cells resulted in the death of most of the cells, which gradually decreased from cortical cells to stellar cells. A few electron-dense granules were precipitated in large vacuoles or in small vesicles in the cytoplasm of cortical parenchyma cells treated with 100 to 400 μM Cd (Fig. 1f). The precipitation increased with increasing concentrations of Cd. At 1000 μM Cd, the abundant electron-dense granules containing Cd formed larger precipitates encircled by a membrane in the vacuoles (Fig. 1e). Small amounts were scattered in the nucleus (Fig. 1e) and cytoplasm (Fig. 1f) of the cortical parenchyma cells. The chloroplasts in the control plants possessed well-developed granal and stromal thylakoids (Fig. 2). Treatment with SA (500 μM) alone did not induce any ultrastructural changes (Fig. 3). Cd treatment at 400 μM caused the thylakoids to swell (Fig. 4A), but 1000 μM Cd caused severe damage (Fig. 4B). On the other hand, in chloroplasts pretreated with SA, the distortion of the thylakoid membrane was effectively suppressed in the 400 μM Cd treatment (Fig. 5A), while at 1000 μM Cd, although the arrangement of the thylakoids was disturbed, severe damage was suppressed by pretreatment with SA (Fig. 5B).

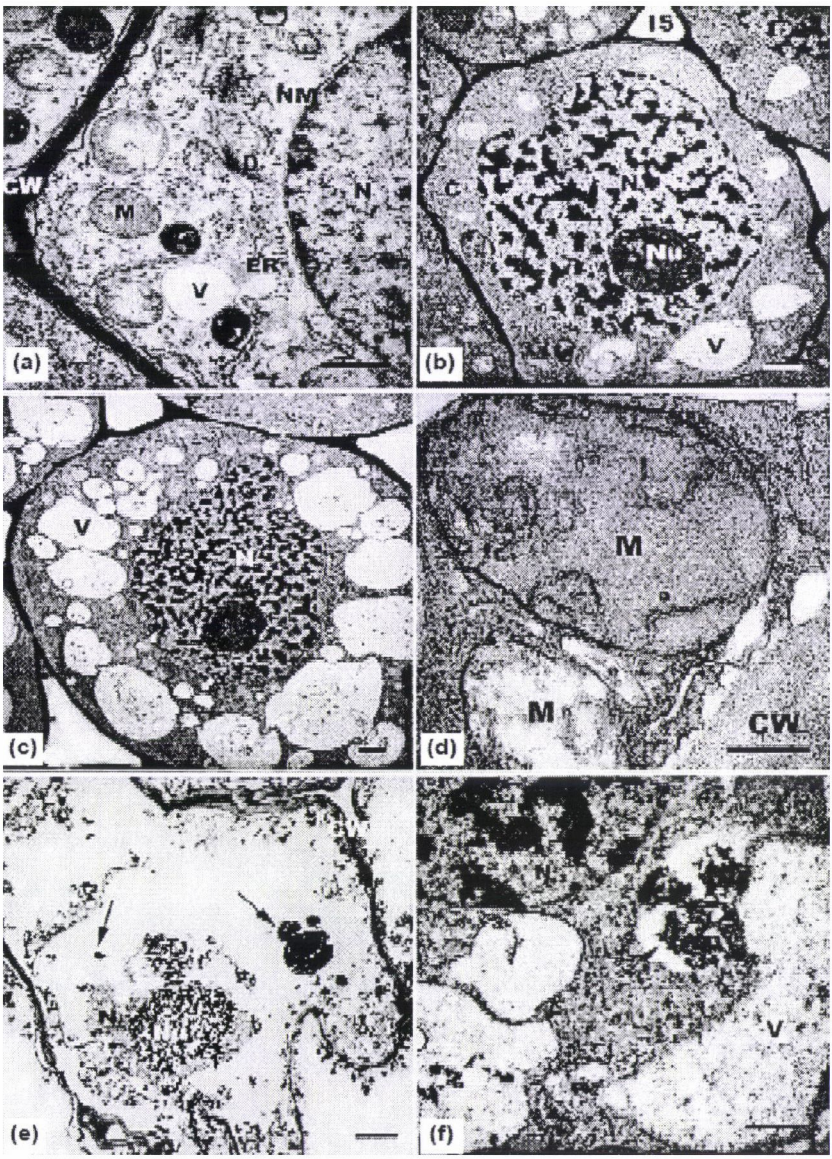

Fig. 1. TEM micrographs showing toxic effects of cadmium on ultrastructure in the root-tip cells of wheat. **(a)** Control cells showing well-developed root-tip cells. **(b)** SA primed cells showing no change compared to the control. **(c–f)** Ultrastructural changes in root-tip cells treated with 100–1000 μM Cd for 9 days. **(c)** Increase in vacuolation (100 μM Cd). **(d)** Reduction in number of mitochondria (400 μM Cd). **(e)** Large precipitates encircled by membranes in the vacuoles and nucleoli containing electron-dense granules (1000 μM Cd). **(f)** A few electron-dense granules distributed in vesicles in the cytoplasm (400 μM Cd). Bar = 0×25 mm **(d)**; 0×5 mm **(a, f)**; 1 mm **(b, c, e)**. Arrows point to electron-dense granules. CW, Cell wall; C, cytoplasm; D, dictyosome; ER, endoplasmic reticulum; IS, intercellular space; M, mitochondria; N, nucleus; NM, nuclear membrane; Nu, nucleoli; V, vacuoles

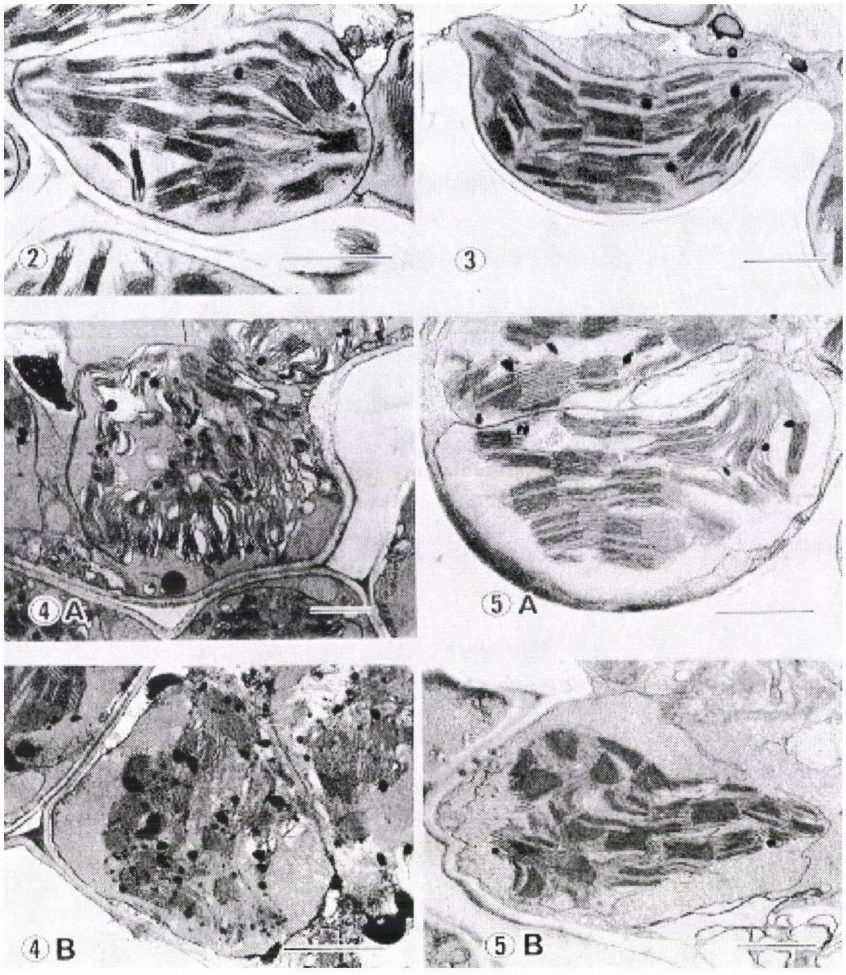

Fig. 2. Ultrastructure of a chloroplast in a control cell. *Fig. 3.* Ultrastructure of a chloroplast in a plant treated with SA alone (500 μM). *Fig. 4.* Ultrastructure of a chloroplast in a plant treated with Cd (A: 400 μM, B 1000 μM). *Fig. 5.* Ultrastructure of a chloroplast in a plant treated with Cd (A: 400 μM, B 1000 μM) after pretreatment with SA (500 μM)

Discussion

SA priming for 20 h resulted in an initial increase in the growth and dry weight of the roots, while in SA-free roots Cd resulted in growth inhibition. Usually, Cd is retained in the roots and only very small amounts are transported to the shoots (Cataldo et al., 1983). The Cd content was higher in SA-free roots than in those primed with SA (Choudhury and Panda, 2004). This differential accumulation of Cd can be considered as potentiating the physiological effect of SA. Cd is known to inhibit plant growth (Moussa, 2004), while the growth-

inducing properties of SA were reported in Cd-treated barley (Metwally et al., 2003) and rice (Choudhury and Panda, 2004). Cadmium toxicity leads to water balance disturbances, manifested by a decrease in RWC, as observed by Costa and Morel (1994). It has been demonstrated that presoaking wheat seeds in 500 μM SA for 20 h before exposure to Cd has a protective effect on RWC. These results are in agreement with the findings of Krantev et al. (2006). The growth inhibition produced by Cd could be due mainly to the effect of this heavy metal on growth and on the photosynthesis rate (Metwally et al., 2003). Cd stress decreased the rate of CO_2 assimilation and photosynthetic efficiency, and led to the degradation of chlorophyll and the inhibition of biosynthesis, which could result in disturbances in the electron transport rates of PSI and PSII, leading to the generation of oxygen free radicals (Moussa, 2004). Treatment with SA increased the pigment contents (Moussa and Khodary, 2003; Krantev et al., 2008). Pretreatment of plants with SA induced a considerable increase in $^{14}CO_2$ assimilation, photosynthetic activity and chlorophyll content, as also reported by Khan et al. (2003), Moussa and Khodary (2003) and Krantev et al. (2006). The activities of both carboxylating enzymes (RuBPC and PEPC) exhibited a strong reduction at all the Cd concentrations applied (Moussa, 2004; Krantev et al., 2006). Pretreatment of wheat plants with SA before exposure to Cd alleviated the inhibitory effect of Cd on these enzyme activities (Moussa and Khodary, 2003; Krantev et al., 2006). Phosphoenolpyruvate carboxylase has been reported to play more than a metabolic role in plants and a non-prevalent form of PEPC could fix CO_2, with malate as the end-product. It has also been proposed that the malate generated in this reaction may function in cell osmoregulation (Harpster and Taylor, 1986). SA application resulted in the activation of PEPC in barley plants (Pancheva et. al., 1996). Rubisco is the primary enzyme of photosynthetic carbon fixation. Cd^{2+}, as a divalent cation, may displace the Mg^{2+} ions which act as activators for Rubisco, resulting in a loss of activity (Wildner and Henkel, 1979). The highest Cd concentration decreased the Rubisco regeneration capacity of the Calvin cycle and photosynthesis (Moussa, 2004). These severe alterations in chlorophyll level, the high extent of lipid peroxidation, the decrease in RuBPC and PEPC activity and CO_2-fixation rates are some of the factors responsible for damage to the photosynthetic process (Krantev et al., 2006; Moussa, 2004). An increase in H_2O_2 production is reported in plants exposed to Cd treatments (Choudhury and Panda, 2004; Krantev et al., 2006). In addition, it was demonstrated that SA pretreatment decreased the MDA accumulation caused by Cd, confirming the role of this compound against oxidation damage (Choudhury and Panda, 2004; Krantev et al., 2006). The results may indicate that an increased ABA level followed by the application of SA and before Cd stress may be responsible for the alleviation of membrane injury under Cd stress conditions (Bandurska and Stroinski, 2005). Free proline accumulation appeared to be a suitable indicator for heavy metal stress. The observed decrease in the proline level in plants grown from SA-pretreated seeds

indicated partial recovery from Cd stress (Krantev et al., 2006). It has been suggested that free proline acts as an osmoprotectant (Delauney and Verma, 1993) and as a metal chelator (Farago and Mullen, 1979). Proline also acts directly as an antioxidant to protect the cell from free radical damage and maintain a more reducing environment, which is favourable for phytochelation synthesis and Cd sequestration (Surasak et al., 2002). Ultrastructural investigations on the root cells of wheat after treatment with Cd indicated that structural alterations and metal accumulation in the cells were dependent on the concentration of the metal. At the ultrastructural level, 100 μM Cd does not cause significant cellular damage to root cells. However, this concentration of Cd causes a greater degree of cell vacuolation, increasing compartmentation in the meristems and cortical parenchyma cells. Sanità di Toppi and Gabbrielli (1999) indicated that vacuolar compartmentalization plays a significant role in Cd detoxification and Cd tolerance, preventing the free circulation of Cd ions in the cytosol and forcing these ions into a limited area. The toxicity symptoms seen in the presence of excessive amounts of Cd may be due to the destruction of all defence systems. This paper provides evidence that at higher concentrations of Cd (400–1000 μM Cd), the toxic symptoms in root cells mainly consist of the disintegration of cell organelles, the disruption of membranes, the withdrawal of the plasma membrane from the cell walls and the formation of multivesiculate bodies in the cytoplasm. Early research reported that the occurrence of electron-dense deposits in vacuoles and the appearance of small vesicles in the cytoplasm seemed to be common features of metal-stressed plants. The presence of metal-bearing granules in the vacuoles and vesicles is related to metal detoxification and the tolerance of plants treated with heavy metals (Rauser and Ackerley, 1987; Nassiri et al., 1997a). The level of Cd increases with an increase in electron-dense granules. A high content of Cd is detected in the vacuoles of cortical cells in differentiating and mature root cells. These results support the findings observed by X-ray microanalysis (Rauser and Ackerley, 1987), and by analytical EM. These results also support the view that the occurrence of electron-dense deposits in vacuoles and vesicles may play an important role in Cd detoxification by maintaining low levels of Cd (Nassiri et al., 1997b). Cd stress decreased the chlorophyll content, and the thylakoids of the chloroplasts were swollen and showed a wavy shape (Pietrini et al., 2003). It is reported that structural changes and the swelling of the thylakoid might be due to a change in the ionic composition of the stroma (Salama et al., 1994). In addition, changes in the thylakoids have been reported to be a typical symptom of oxidative stress (Hernandez et al., 1995).

One important role of SA in inducing resistance to various environmental stresses is manifested by its ability to express genes that code for pathogenesis-related proteins or defence-related enzymes (Merkouropoulos et al., 1999). Also, SA could form a complex with Cd, which might provide Cd tolerance (Choudhury and Panda, 2004).

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EXOGENOUS ASCORBIC ACID OR THIAMINE INCREASES THE RESISTANCE OF SUNFLOWER AND MAIZE PLANTS TO SALT STRESS

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Increasing NaCl levels retarded the net photosynthetic rate, biosynthesis of photosynthetic pigments and membrane integrity of maize and sunflower seedlings; a serious effect was exhibited when NaCl was applied at high concentration. On the other hand, the K^+ efflux increased at increasing NaCl levels. In addition, the various salt levels induced considerable variations in the concentrations of sodium, potassium, calcium and magnesium. The vitamins applied were generally effective in partially or completely countering the inhibitory effects of salt stress on net photosynthetic rate, pigments biosynthesis and membrane integrity, exerting a stimulatory action on these parameters, especially in plants subjected to moderate and low salinity levels. The leakage of K^+ was reduced by the application of both ascorbic acid (AsA) and thiamine (B_1). Soaking the seeds of salt-stressed plants in AsA or B_1 had a favourable effect on the accumulation of certain ions and antagonized or ameliorated the inhibitory effect of salt stress.

Key words: ascorbic acid, calcium, magnesium, membrane integrity, sodium chloride, pigments, potassium, sodium

Abbreviations: AsA, ascorbic acid; B_1, thiamine; AOS, active oxygen species; SOS, salt overly sensitive; PL, pyridoxal; PdxK, pyridoxal kinase; PM, pyridoxamine; PN, pyridoxine; L-GL, L-galactone-γ-lactone; MDA, monodehydroascorbate.

Introduction

Excess minerals in soils can be a major problem in arid and semi-arid regions because rainfall is insufficient to leach the mineral ions from the soil. Worldwide, up to 20% of the irrigated arable land is already salt-affected and this portion is still expanding (Mühling and Läuchli, 2001). Salt stress affects plant growth and development in many different ways. Excess salt causes ion toxicity inside the cell. High concentrations of salt in the root medium also create hyperosmotic stress, which impedes water absorption and transport. Secondary stresses such as nutritional imbalance and oxidation often occur as a

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consequence of ion toxicity and hyperosmotic stress (Zhu, 2001). Plants respond to salt stress by changing their gene expression pattern, metabolic activity, and ion and water transport in order to minimize stress damage and to re-establish ion and water homeostasis (Hasegawa et al., 2000). In addition, high exogenous salt concentrations cause the production of active oxygen species (AOS) (Cramer et al., 1994). AOS can damage essential membrane lipids as well as protein and nucleic acids (Noctor and Foyer, 1998). Levels of AOS in plant cells are normally controlled by protective antioxidant activity. However, under environmental stress, AOS production may increase and protective activity may then become inadequate. Reactive free oxygen radicals are assumed to be involved in the oxidation of ascorbic acid to dehydroascorbic acid, leading to a reduction in the ascorbic acid content of the plant (Singh et al., 2006). Also, generalized oxidative stress may reduce the levels of thiamine, thiamine phosphates and the enzymes that act on thiamine. The decline in thiamine or thiamine phosphates and thiamine-dependent enzymes could exaggerate oxidative stress and lead to neuro-degeneration (Gibson and Zhang, 2002). In addition to the important role of thiamine in plant biosynthetic pathways, it may also induce the accumulation of pathogenesis-related protein in a salicylic acid (SA)-dependent pathway and the enhancement of disease resistance in tobacco, *Arabidopsis*, cucumber and rice (Malamy et al., 1996; Ahn et al., 2007).

The present work thus investigated salt-stress induced changes that may inhibit the photosynthesis, photosynthetic pigments, membrane integrity and mineral content of salt-treated maize (C_4) and sunflower (C_3) plants. The interactive effects of AsA or B_1 and salt stress were considered to estimate whether the two vitamins were able to ameliorate salt stress-induced changes.

Materials and methods

Plant material

Maize (*Zea mays* L.) and sunflower (*Helianthus annuus* L.) plants were used in the experiments. In preliminary tests seeds were surface-sterilized and soaked in different concentrations (100, 150, 200, 250, 300, 350, 400, 450 and 500 μM) of exogenously applied vitamins for different times (4, 6, 8, 10, 12 and 24 h). The best concentrations were found to be 300 μM ascorbic acid (AsA; vitamin C) and 150 μM thiamine (B_1; vitamin B_1) and the optimum time 6 hours, so these were applied to test the role of vitamins in alleviating the adverse effects of salinity on the test plants. Other grains and seeds were soaked in distilled water and used as a control. The seeds were then dried at room temperature for 48 hours. The seeds were sown five to a pot in plastic pots (11.5 cm in diameter, 10 cm deep) lined with polyethylene bags and filled with 1 kg of a 1:1 (v/v) mixture of sieved air-dried clay and sand. The pots were then irrigated with various saline solutions to reach the desired salinization level (50, 100, 150 and 200 mM NaCl). The water content of the soil was adjusted to near field capacity and the whole system (pot, soil and polyethylene bag) was weighed. Thereafter, the pots were irrigated every other day with water to reach field capacity. Some were left untreated (no salt or vitamins) as an absolute control, while salinized plants without vitamins were regarded as reference control plants. The plants were left to grow under these conditions for 30 days.

Cell membrane stability

Cell membrane stability was determined according to the method of Blum and Ebercon (1981). Ten fresh leaf discs (1 cm in diameter) were taken from each pot and washed three times with deionized water to remove surface-adhered electrolytes. They were then placed in 30 cm^3 deionized distilled water for 24 h at 10°C and the electrical conductivity of the bathing solution was measured at 25°C. Following measurements, the leaf discs were autoclaved for 15 min, cooled to 25°C, and the electrical conductivity of the bathing solution was measured for a second time. The degree of injury was calculated according to the equation:

$$\text{Percentage injury (\%)} = (T_1/T_2)/(1-C_1/C_2)\ .100$$

where T_1 and T_2 are the first and second conductivity measurements (before and after autoclaving) for each treatment and C_1 and C_2 the first and second conductivity measurements for the control. A Carl Zeiss flame photometer was used for the determination of K^+ (Williams and Twine, 1960).

Photosynthetic pigments and net photosynthesis

The contents of chlorophyll a, b and carotenoids were determined spectrophotometrically (Metzner et al., 1965). Net photosynthetic rate (oxygen evolution) was followed manometrically using disks of leaf tissue exposed to 25°C and irradiance of 5.9 Wm^{-2} (40 *WEF* lamps) using the Warburg apparatus type VL 85 (Umbreit et al., 1959).

Mineral composition

Sodium and potassium were determined by the flame photometer method (Williams and Twine, 1960) and calcium and magnesium by the versene titration method (Schwarzenbach and Biedermann, 1948).

Statistical analysis

The data were subjected to a one-way analysis of variance (ANOVA) using the SPSS statistical package. Duncan's multiple range test ($P<0.05$) was used for comparison of the means.

Results

Membrane stability

Membrane stability showed a highly significant decrease with increasing salinity levels in both maize and sunflower plants (Fig. 1). The inhibitory effects of salt stress on the membrane stability of maize and sunflower plants were partially alleviated by seed soaking in 300 µM AsA or 150 µM B_1 (Fig. 1).

K^+ efflux is a good stress index as it reflects the degree of plant injury by salt. The K^+ efflux of the maize and sunflower plants increased in response to salt stress (Fig. 2). However, treatment with AsA or B_1 exerted a retarding effect on the K^+ efflux in salt-stressed maize and sunflower leaves (Fig. 2).

Photosynthetic pigments and net photosynthesis

The data presented in Figure 3 indicate that the biosynthesis of photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) and the net photosynthetic rate of both maize and sunflower plants were affected by salt stress and by their interaction with each of the two vitamins applied (AsA and B_1). The data show that stress had an inhibitory effect on the biosynthesis of the pigment fractions and on the net photosynthetic rate in maize and sunflower

leaves. On the other hand, soaking the seeds of maize and sunflower in AsA or B_1 was generally effective in alleviating, partially or completely, the inhibitory effects of salinization treatments on pigment biosynthesis and net photosynthetic rate.

Mineral composition

Salinity induced a considerable accumulation of sodium in the shoots and roots of maize and sunflower plants. This accumulation was more prominent at moderate and higher salinization levels (Fig. 4), and was much higher in the shoots than in the roots, whatever the salinization level used. In most cases, vitamin treatment retarded the accumulation of sodium in the shoots and roots of salinized test plants (Fig. 4), but the retarding effect was more pronounced at lower salinization levels. On the other hand, increasing soil salinity resulted in a considerable decrease in the K^+ content in the shoots and roots of maize and in the roots of sunflower plants (Fig. 5), particularly at moderate and higher salinization levels. In sunflower shoots, however, the data clearly demonstrate that salinity stimulated the accumulation of K^+ up to the highest salinization level used. Vitamin treatment generally resulted in a marked increase in the K^+ content in the shoots and roots of maize and sunflower plants compared with those of plants treated with NaCl only.

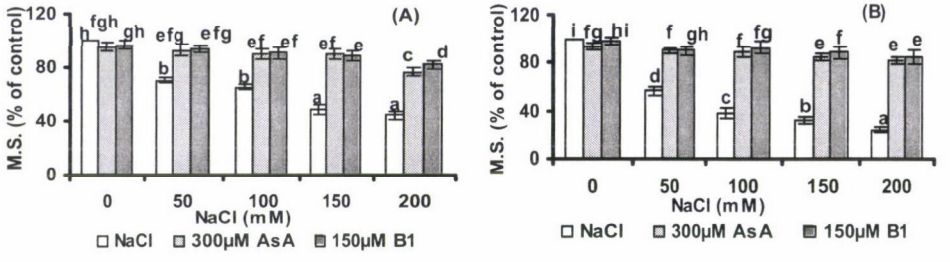

Fig. 1. Interactive effects of salinity and vitamins on the membrane stability (M.S.) of maize (A) and sunflower (B) plants. The results are means (± SE) of five replicates. Bars carrying different letters are significantly different at $P<0.05$

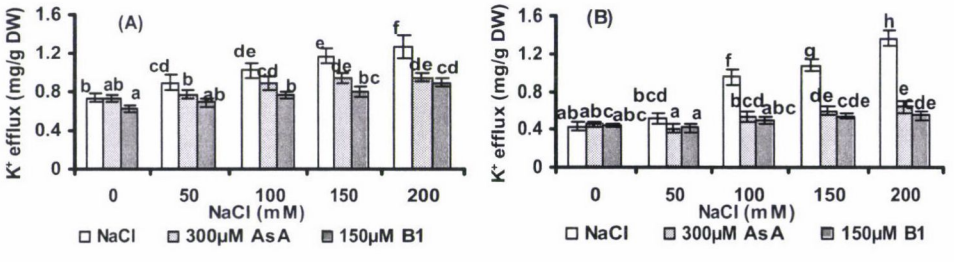

Fig. 2. Interactive effects of salinity and vitamins on the K^+ efflux of maize (A) and sunflower (B) plants. The results are means (± SE) of five replicates. Bars carrying different letters are significantly different at $P<0.05$

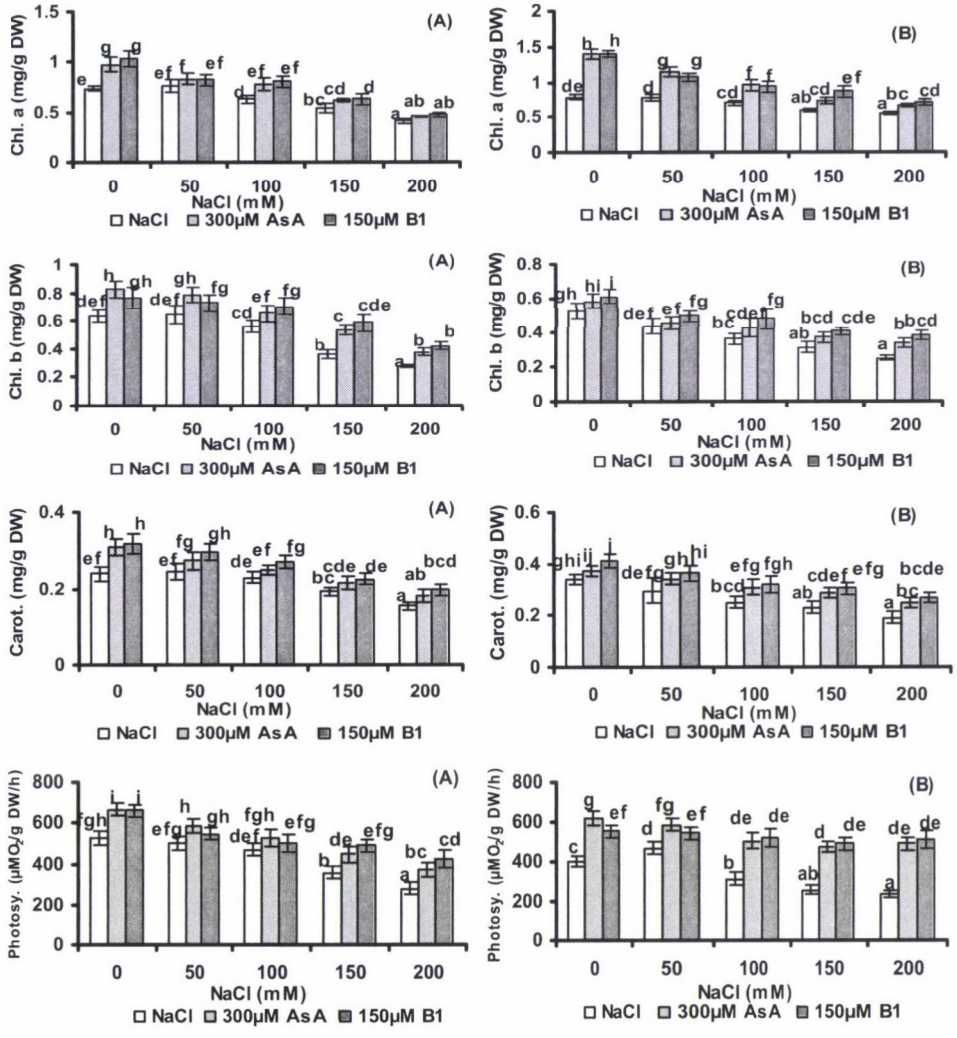

Fig. 3. Interactive effects of salinity and vitamins (AsA or B_1) on the photosynthetic pigments [chlorophyll a (chl.a), chlorophyll b (chl.b) and carotenoids (carot.)] and net photosynthetic rate (Photosy.) of maize (A) and sunflower (B) plants. The results are means (± SE) of five replicates. Bars carrying different letters are significantly different at $P<0.05$.

The calcium and magnesium contents in the shoots and roots of maize and sunflower plants were markedly elevated by a rise in the salinization level (Figs. 6 and 7). In general soaking the seeds in AsA induced an increase in the Ca^{2+} content in the shoots and roots of maize and sunflower plants, while soaking in B_1 led to a pronounced reduction in Ca^{2+} in the shoots of both crops compared with the salinized control. However, both AsA and B_1 treatment stimulated the accumulation of Mg^{2+} in the shoots and roots of sunflower at most salinization levels and in the roots of maize plants. In most cases, vitamin treatment did not induce any marked change in the Mg^{2+} content in maize.

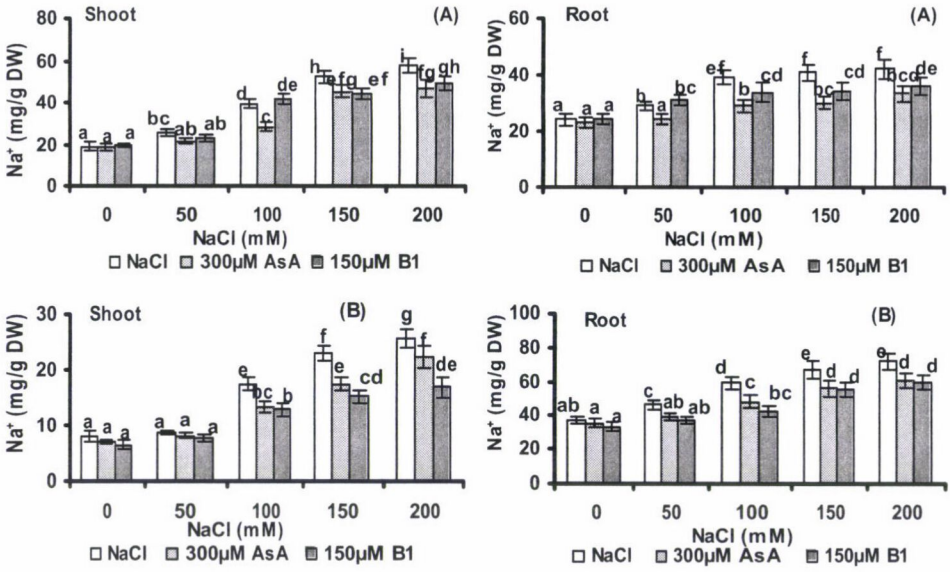

Fig. 4. Interactive effects of salinity and vitamins (AsA or B_1) on the Na^+ content of maize (A) and sunflower (B) plants. The results are means (± SE) of five replicates. Bars carrying different letters are significantly different at $P<0.05$.

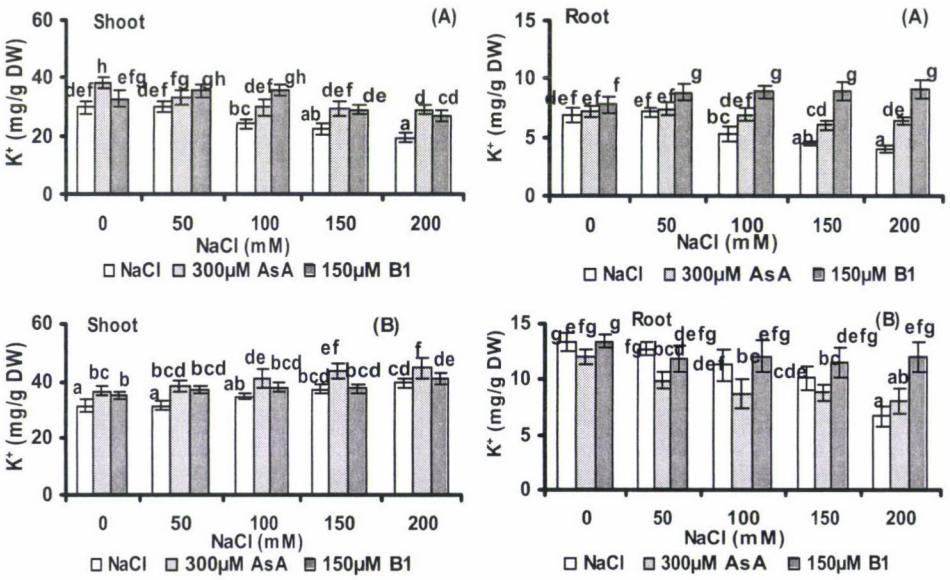

Fig. 5. Interactive effects of salinity and vitamins (AsA or B_1) on the K^+ content of maize (A) and sunflower (B) plants. The results are means (± SE) of five replicates. Bars carrying different letters are significantly different at $P<0.05$

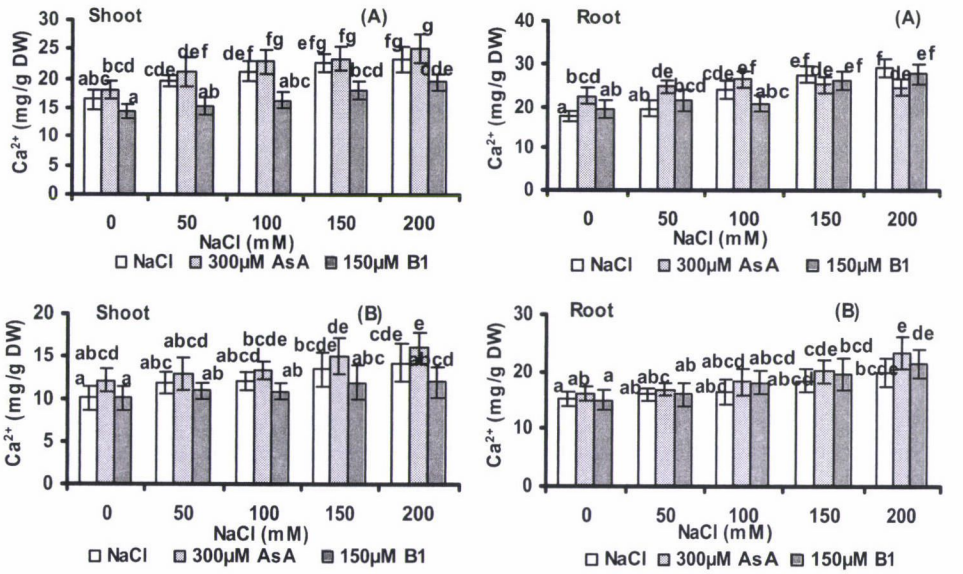

Fig. 6. Interactive effects of salinity and vitamins (AsA or B_1) on the Ca^{2+} content of maize (A) and sunflower (B) plants. The results are means (± SE) of five replicates. Bars carrying different letters are significantly different at $P<0.05$.

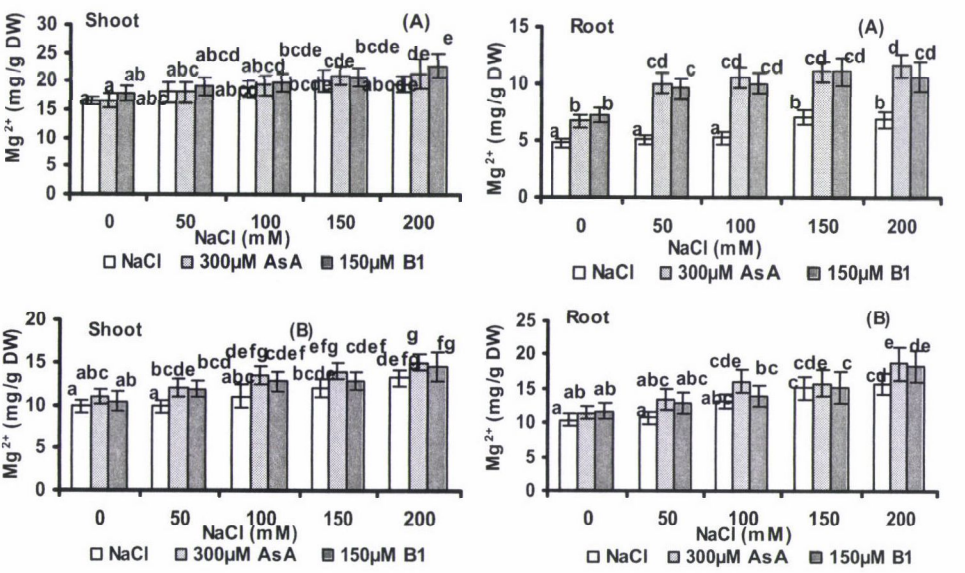

Fig. 7. Interactive effects of salinity and vitamins (AsA or B_1) on the Mg^{2+} content of maize (A) and sunflower (B) plants. The results are means (± SE) of five replicates. Bars carrying different letters are significantly different at $P<0.05$

Discussion

The plasma membrane plays a crucial role when plants are forced to respond to environmental stimuli. The lipid composition of the plasma membrane controls membrane permeability and its efficiency as a semi-permeable barrier (Meharg, 1993). Also, plasma membranes are injured more seriously as stress intensifies, leading to an increase in the electrolyte leakage rate. Stressed plants exhibit significantly low values of percentage integrity, which is based on the relationship between cellular constituents and the leakage of K^+. Vitamins ameliorated this effect, improving membrane integrities. Accordingly, Hubac et al. (1989) stated that the integrity of the cellular membrane structure and the preservation of the membrane are of primary importance in the tolerance of water stress. These biochemical and biophysical changes in the membrane were stimulated by oxygen free radical treatment (Kendall and McKersie, 1989). Active oxygen species (AOS) react with membrane lipids causing their peroxidation, which leads to membrane damage (Noctor and Foyer, 1998). AsA is directly involved in the ascorbate peroxidase-mediated detoxification of H_2O_2 and indirectly in that of lipid peroxides. Lipid peroxides and lipid radicals are quenched by tocopherol. AsA is the electron donor in the reduction of oxidized tocopherol (Noctor and Foyer, 1998). Shalata and Neumann (2001) concluded that root treatment with exogenous AsA can greatly increase the capacity of tomato seedlings to survive the other lethal effects of a 9 h exposure to severe salt stress (300 mM NaCl). The increase in plant resistance to salt stress was associated with the antioxidant activity of AsA and the partial inhibition of salt-induced increases in lipid peroxidation by AOS. Also, thiamine is an antioxidant (Frederiks et al., 1999), normalizing lipid peroxidation levels and elevating glutathione reductase activity (Tolstykh and Khmelevskii, 1991). Vitamin B_1 interacts with free radicals and is oxidized to thiochrome and thiamine disulphide. The antioxidant effect of B_1 is probably related to the successive transfer of 2 H^+ from the NH_2 group of the pyrimidine ring and H^+ from the thiazole ring to reactive substrates (Lukienko et al., 2000). B_1 also reduces lead-induced endogenous lipid peroxidation and diminishes the histopathological lesions in rat hepatic and renal tissues (Senapati et al., 2000).

K^+ is the most abundant cation in plant cells and it is involved in many physiological processes (Glass, 1983). K^+ transport across the plasma membrane is due to the operation of both active and passive systems and its leakage is usually used as a criterion for the stability and integrity of membranes. In this work, the leakage of K^+ was assessed under salinity stress. Membrane disorders induced by stress conditions are of the elastic type. Sreenivasulu et al. (2000) concluded that salinity caused stronger electrolyte leakage from sensitive foxtail millet seedlings. The application of AsA or B_1 was generally effective in partially antagonizing the stimulatory effect of salt stress on the leakage of K^+. The activation of plant growth in salt-stressed plants after seed soaking in AsA

or B_1 suggests that these vitamins activate the antioxidant enzymes responsible for the breakdown of H_2O_2 during growth under salinity stress. In this context, McKersie et al. (1999) pointed out that one possibility was that AsA inhibited the stress-induced increase in the leakage of essential electrolytes following peroxidative damage to plasma membranes. Guo et al. (2005) concluded that AsA and its precursor increased the resistance of rice seedlings to chilling and drought.

The decline in the biosynthesis of photosynthetically active pigments and in the net photosynthetic rate in maize and sunflower leaves as a function of successive increases in the salinization level may be due to the direct effect of salt on stomatal resistance via a reduction in guard cell turgor, leading to a reduction in intercellular CO_2 partial pressure (Dionisio-Sese and Tobita, 2000). Although other investigators also attributed the inhibition of CO_2 assimilation by salinity to reduced stomatal conductance (Yeo et al., 1985), the importance of the ion effect on the enzymatic factors of photosynthesis cannot be entirely ruled out. Also, since rice is a salt-sensitive crop, where only a minor substitution of K^+ by Na^+ is possible (Marschner, 1995), the excess Na^+ in salt-sensitive cultivars displaces the essential cations from the membrane structure, leading to a change in permeability, or, in the case of the chloroplast structure, to the swelling and disorganization of the grana (Flowers et al., 1985). In addition, water stress is known to inhibit the photosynthetic activity in tissues due to an imbalance between light capture and its utilization (Foyer and Noctor, 2000). The beneficial effects of the two vitamins applied in partially or completely mitigating the adverse effects of salt stress on photosynthetic pigments and the net photosynthetic rate were clearly exhibited by the test plants. This mitigation of the harmful effects of salt stress could be directly attributed to the role of the applied vitamins in enhancing photosynthetic activity and chlorophyll biosynthesis. These results are in agreement with Choudhury et al. (1993), who attributed positive effects to vitamins in stabilizing the photosynthetic pigments and photosynthetic apparatus and protecting them from oxidation. This is in agreement with Muckenschnabel et al. (2002), who concluded that the damage to the photosynthetic apparatus occurs after the depletion of endogenous antioxidants. AsA is water-soluble and also has an additional role in protecting or regenerating oxidized carotenoids or tocopherols (Imai et al., 1999). AsA is a major metabolite in chloroplasts of higher plants and represents about 10% of the soluble carbohydrate pool in leaves (Noctor and Foyer, 1998). Also, in tobacco (*Nicotiana sylvestris*), a thiamine-deficient mutant does not produce chlorophyll a and b and carotenoids (McHale et al., 1988). In addition, data from other plants suggest that the synthesis of β-carotene occurs via a metabolic pathway involving the thiamine-dependent conversion of pyruvate to acetyl-CoA by plastid pyruvate dehydrogenase (Schulze-Siebert and Schultz, 1997).

The major ions involved in salt stress signalling include Na^+, K^+, H^+ and Ca^{2+} (Mahajan and Tuteja, 2005). It is the interplay of these ions which

maintains homeostasis in the cell. The application of NaCl induced Na^+ accumulation in the shoots and roots of the tested plants. The disruption of the ionic equilibrium and the influx of Na^+ dissipate the membrane potential and facilitate the uptake of Cl^- down the chemical gradient. Na^+ is toxic to the cell metabolism and has a deleterious effect on the functioning of some enzymes (Niu et al., 1995). High concentrations of Na^+ cause osmotic imbalance, membrane disorganization, reduction in growth, inhibition of cell division and expansion. In addition, high Na^+ levels lead to a reduction in photosynthesis and the production of reactive oxygen species (Yeo, 1998). Increasing the soil salinity resulted in a considerable decrease in K^+ contents in the shoots and roots of maize and the roots of sunflower plants. As common proteins transport Na^+ and K^+, Na^+ competes with K^+ for intracellular influx (Blumwald et al., 2000). Many K^+ transport systems have some affinity for Na^+, i.e. they are Na^+/K^+ symporters. Thus, external Na^+ negatively affects the intracellular K^+ influx. However, moderate salinity treatments promoted the accumulation of K^+ in sunflower shoots. This accumulation may contribute to the phenomenon of osmotic adjustment (Bolarin et al., 1995). The maintenance of adequate net uptake of K^+ at high Na^+ concentrations is important.

High salinity results in increased cytosolic Ca^{2+}, which is transported from the apoplast and intracellular compartments (Knight et al., 1997). It was observed in the present work that increasing the concentration of NaCl from 50 to 200 mM increased both the Ca^{2+} and Mg^{2+} levels in the shoots and roots of the test plants. This transient increase in cytosolic Ca^{2+} initiated the stress signal transduction leading to salt adaptation. Carter et al. (2005) found that the Mg^{2+}, Na^+ and Cl^- concentrations increased with increasing salinity in two cultivars of *Celosia argentea*, which is in agreement with the present results.

On the other hand, soaking seeds in AsA or B_1 induced a significant decrease in the accumulation of Na^+ in most cases, while promoting the absorption of K^+, Ca^{2+} and Mg^{2+}. However, B_1 induced a pronounced reduction in the Ca^{2+} content in the shoots of both maize and sunflower plants. Limiting Na^+ entry into the cell is probably one of the most important mechanisms to maintain a low Na^+ concentration in the cytosol. Evidence suggests that plant ion channel activities are modulated by a variety of cellular factors, including secondary messengers such as cytosolic calcium, pH and nucleotides. Ion channels are known to interact with signalling proteins (Zimmermann et al., 1999). Direct ligand binding to the channel proteins is an important mechanism for the regulation of transport activities. The availability of the whole sequence of the *Arabidopsis* genome led to the discovery of NHX vacuolar sodium-proton antiporter genes, which improve plant resistance to salt stress by increasing sodium sequestration into the vacuole. One of the most powerful strategies to identify the determinants of salt tolerance is a forward genetic approach. Initial screening for salt-overly-sensitive (*SOS*) mutants identified three genes: SOS_1, a putative sodium transporter, which points again to the importance of the control

of solute transport through the membrane; and SOS_2 and SOS_3, which reveal the implication of calcium and kinase signalling during salt stress. Shi et al. (2002) identified a molecular lesion in a new *SOS* mutant, SOS_4, which shows that plants also require their own vitamins to manage salt stress. Mutant SOS_4 seedlings are hypersensitive to several alkali ions, including sodium, its more toxic analogue lithium, and potassium. Mutations in SOS_4 also result in the over-accumulation of sodium. This suggests a possible defect in the regulation of cation uptake in SOS_4 mutants. Surprisingly, the gene impaired by SOS_4 mutations encodes a functional pyridoxal kinase (PdxK). Pyridoxal kinases activate vitamin B_6 (pyridoxal, Pdx) to form the versatile coenzyme pyridoxal phosphate (PdxP), which is an essential cofactor for many enzymes implicated in the amino acid metabolism. It would thus be expected that a defect in the gene encoding PdxK should cause a lethal phenotype. However, it was reported that the hydroxymethylpyrimidine kinase of *E. coli*, involved in B_1 biosynthesis, has kinase activities for pyridoxal (PL), pyridoxamine (PM) and pyridoxine (PN) (Mizote and Nakayama, 1989). Although one gene in *Arabidopsis* encoding a putative hydroxymethylpyrimidine kinase shows only 29% identity and 49% similarity to SOS_4 over a stretch of 107 amino acids, it is possible that this protein or even other proteins in *Arabidopsis* function as PN kinase in the salvage pathway of PLP biosynthesis. Therefore, PLP biosynthesis in SOS_4 is probably disrupted incompletely. It is possible that PLP production in SOS_4 mutants is unbalanced, resulting in the inappropriate regulation of PLP-dependent enzyme or ion transporters (Shi et al., 2002).

It can thus be concluded that seed soaking in AsA or B_1 can remarkably increase the capacity of maize and sunflower seedlings to resist salt stress. The increase in plant resistance to salt stress may be associated with the antioxidant activity of AsA and B_1. In addition, B_1 may re-establish ion homeostasis by maintaining a relatively high K^+ concentration and low Na^+ concentration in the cytosol of tested plants.

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GROWTH, NODULATION AND N_2 FIXATION OF *Sesbania aculeata* GROWN ON SOIL AMENDED WITH PHOSPHOGYPSUM

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The impact of five rates of phosphogypsum (PG) (0, 5, 10, 20 and 40 t/ha) on the growth, nodulation and N_2 fixation of dhaincha (*Sesbania aculeata* Pers.) was evaluated in a pot experiment, using sorghum (*Sorghum bicolor* L.) as a reference crop. N_2 fixation by the legume crop was measured using the ^{15}N isotope dilution method. The dry matter content of sorghum doubled when the soil was supplied with the lowest rate of PG (5 t/ha). For sesbania, the highest rate of PG (40 t/ha) was found to have a significant effect on the dry matter yield. PG had a beneficial effect on phosphorus (P) accumulation in both plant species, particularly in the nodules of sesbania. The beneficial effect of PG on nodulation and N_2 fixation was more pronounced than on the host plant growth. The highest value of N_2 fixation (67%) was obtained following the addition of 10 t PG/ha, whereas it was only 35% in the control treatment (PG0). The amount of fixed N_2 doubled when the soil was supplied with PG, particularly in the PG10 treatment. The concentration of fluoride (F^-) in the shoots of both plant species was less than 10 mg/kg. In conclusion, PG improved nodulation, N_2 fixation and P availability in the legume species *S. aculeata* with minimal soil N uptake.

Key words: phosphogypsum, N_2 fixation, *Sesbania aculeata*, *Sorghum bicolor*, ^{15}N

Introduction

The production of phosphoric acid by the acidulation of rock phosphate with sulphuric acid produces phosphogypsum (PG) as a by-product. Phosphogypsum is primarily composed of $CaSO_4$ and appears to be an environmentally safe source of sulphur (S) and calcium (Ca) for crops, as well as for other uses (Alcordo and Rechcigl, 1993). Phosphogypsum is usually stored in piles close to fertilizer factories. The escape of PG into the environment as the piles are exposed to weathering processes may lead to chemical and radioactive contamination. This is because PG contains a number of harmful elements, such as F^-, other trace elements, and the radioactive materials ^{226}Ra and ^{210}Po

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(Rutherford et al., 1994; Burnett et al., 1999; Al-Masri et al., 2004; Al-Masri and Al-Bich, 2002). In Syria, more than one million tons are available as a solid by-product of the phosphoric acid industry (Al-Oudat, 2000). As the accumulation of PG in piles may represent an environmental hazard to surrounding areas, its utilization for improving plant growth and agricultural production would be of double benefit. The use of PG to amend the physical and chemical properties of soils has been widely reported (Alcordo and Rechcigl, 1993; Rutherford et al., 1994; Al-Oudat et al., 1998; Al-Karaki and Al-Omoush, 2002). PG increased the productivity of plants (e.g. clover and barley) without increasing the radioactivity in their productive and vegetative parts (Al-Oudat et al. 1998). Alcordo and Rechcigl (1993) highlighted the scarcity of studies on the use of PG in agriculture. They reported that PG did not appear to constitute environmental hazards at the rates normally used in agriculture, and could be considered an effective way of increasing plant productivity. Moreover, the use of PG in agriculture may offer an economically attractive and ecologically sound means of reducing the environmental hazards caused by its storage in piles near fertilizer factories.

Legumes have long been recognized as important components of crop rotations and intercrop systems. Increasing the biological nitrogen fixation of legumes is considered to be a significant challenge. The enhancement of N_2 fixation could be achieved in several ways, including the selection and engineering of rhizobia, the selection and breeding of legume genotypes and the improvement of management practices. Several nutrients essential for the growth of plants or bacteria play a specific role in nodulation and/or N_2 fixation.

Dhaincha (*Sesbania aculeata* Pers.) is a rapidly growing leguminous plant, and is adapted to a variety of soil conditions, ranging from waterlogged to saline, and from sandy to clayey soil (Sandhu and Haq, 1981; Kurdali and Al-Ain, 2002). It is used for green manuring (Kurdali, 2004) and fodder production (Zarkawi et al., 2003), besides its use in intercropping systems with non-legume species (Kurdali et al., 2003). Information on the effect of PG on the growth and N_2 fixation of legume plants is scarce, but knowledge on the response of plant growth following the addition of PG is of primary importance, from both the agronomic and environmental point of view. Therefore, this work was undertaken to study the impact of PG on the growth, nodulation and N_2 fixation of dhaincha (*Sesbania aculeata* Pers.) using sorghum (*Sorghum bicolor* L.) as a reference crop. N_2 fixation by the legume crop was measured using the ^{15}N isotope dilution method.

Materials and methods

Soil properties and phosphogypsum amendment treatments

The experiment was conducted during the summer season (June–August) at Der-Alhajar Research Station, located south east of Damascus, Syria (33°21' N, 36°28' E) at 617 m above sea level. The average minimum temperature in winter is 1.3°C in Jan., while it increases to an average of 36°C during August. The experiment was conducted in pots, each containing 11.2 kg of air-dried soil, passed through a 3-mm sieve. Its texture was sandy clay (50.4% sand, 13.1% silt and 36.5%

clay) and classified as an Aridisol. Subsamples of the soil were taken before planting for the determination of physical and chemical soil properties. The bulk density was 1.3 g/cm^3; pH 7.33; Ec_e 1.37 dS/m; organic matter 0.48%; ionic content (meq/L) Cl^- 3.79, HCO_3^- 2.4, SO_4^{2-} 1.99, Ca^{2+} 1.6, K^+ 0.07, Na^+ 4.9, Mg^{2+} 1.99; $CaCO_3$ 14.67% (meq/100 g) 18.87; available P (Olsen) 3.72 mg/kg; NO_3^- 29.5 mg/kg; NH_4^+ 17.5 mg/kg.

Phosphogypsum samples were collected in plastic bags from different locations in the disposal area near the phosphoric acid factory in Homs (180 km N of Damascus). Equivalent rates of 0, 5, 10, 20 and 40 t/ha (abbreviated as PG0, PG5, PG10, PG20 and PG40, respectively) of air-dried PG were mixed thoroughly with the soil of each pot. The actual amounts of added phosphogypsum were 0, 20, 40, 80 and 160 g per pot in the respective treatments.

Phosphogypsum properties

The analysis of PG showed the presence of 142, 10.6 and 0.9 mg/kg of P_2O_5, NH_4^+ and NO_3^-, respectively. The pH of the solution was 3.54. Dissolving PG in distilled water released 23.1 mmol/L of Ca^{2+}, 17.0 mmol/L of SO_4^{2-}, 8.9 mmol/L of Cl^- and 0.4 mmol/L of HCO_3^-, besides traces of Mg^{2+} and Na^+ (3.29 and 0.43 mmol/L, respectively). The dissolution of these ions in the distilled water raised its EC to 2.92 dS/m. Radiochemical analyses by gamma spectrometer showed that the specific activities of ^{224}Ra, ^{226}Ra, ^{228}Ra, ^{210}Po and ^{234}Th in the PG samples were 2.8±0.7, 245±10, <3.5, 240±30 and 24±10 Bq/kg, respectively.

Planting procedures and ^{15}N application

Seeds of *Sesbania aculeata* Pers. and *Sorghum bicolor* L., as a non-fixing reference crop, were planted in a total of 20 pots each, arranged in a randomized complete block design with four replicates, and set outdoors under natural climatic conditions. The seedlings of each plant species were thinned to four plants per pot when the cotyledons appeared. The pots were weighed every three days, and watered to maintain soil water content at around 60% of field capacity throughout the experimental period. A 500 mL urea solution obtained from the International Atomic Energy Agency, equivalent to 20 kg N/ha or 9.6337 atom % ^{15}N excess, was uniformly applied to both species at planting.

Plant harvest and analysis

Plants of both species were harvested 75 days after planting. Shoot, roots and nodules were dried to constant weight at 70°C and milled to a fine powder (0.5 mm). The samples were digested using the Kjeldahl procedure for the determination of total N. The $^{15}N/^{14}N$-isotope ratio was determined by emission spectrometry (Jasco-150, Japan). The N fraction derived from the atmosphere (%Ndfa) was calculated using the equation of Fried and Middelboe (1977). The phosphorus content was determined in the separate organs (shoots, roots and nodules) of both plant species by dry ashing and measured colorimetrically using a spectrophotometer (Thermo Spectronic, UK). In addition, the fluoride (F^-) concentrations were determined using the selected electron technique. Since there was insufficient nodule mass left, F^- determination was carried out using the remaining nodules collected from all the replicates. The above determination was considered as one analysis for each treatment, except for PG0, as the sample was not adequate.

The data were subjected to analysis of variance (ANOVA), and means were compared using the Least Significant Difference (LSD) test at a probability level of $P<0.05$.

Results

Effect of PG on dry matter yield

The shoot dry matter of *Sesbania aculeata* significantly increased by 30% compared to the control when the soil was supplied with 40 t/ha of phosphogypsum (PG) (Table 1). However, PG did not result in any significant increase in the root dry matter. The effect of PG on the dry matter yield of

sesbania was more pronounced on nodules than on the other plant parts (Table 1). In comparison with the control treatment, the nodule dry matter increased by 28, 52, 55 and 77% as a result of adding 5, 10, 20 and 40 t/ha of PG, respectively.

In sorghum, the application of PG to the soil resulted in a significant increase in the shoot and root dry matter yield (Table 2). The percentage increments in dry matter yield over the control treatment were 86, 52 and 62% in the shoots, and 118, 114 and 68% in the roots, for the PG5, PG10 and PG20 treatments, respectively. However, no significant effect was observed in the PG40 treatment.

Effect of PG on N content and uptake

The nitrogen content in the shoots and roots of sesbania plants did not significantly differ between the PG treatments. However, PG had a significant effect on the %N in the nodules (Table 3). The pattern of N uptake in the sesbania plants was relatively similar to that of dry matter yield (Table 1). N uptake in shoots significantly increased by 33% in the PG40 treatment. The nodule N yield relatively doubled in all PG treatments compared with the control, with average percentage increments over the control of 72, 92, 93 and 125% in the PG5, PG10, PG20 and PG40 treatments, respectively.

Table 1
Dry matter yield, N uptake and P uptake in *Sesbania aculeata* as affected by different rates of phosphogypsum (PG)

Treatments	Shoots	Roots	Nodules	Whole plant
Dry matter yield (g/pot)				
PG0	14.63±1.37b	10.00±0.20a	0.65±0.07b	25.27±1.54b
PG5	16.00±0.93b	10.38±0.23a	0.83±0.15ab	27.20±1.04ab
PG10	16.50±0.93ab	10.88±0.62a	0.99±0.11a	28.37±1.07ab
PG20	17.25±1.13ab	10.25±0.14a	1.01±0.07a	28.51±1015ab
PG40	19.25±0.66a	9.75±0.32a	1.15±0.12a	30.15±0.87a
LSD 0.05	3.11	NS	0.33	3.48
Total N (mg/pot)				
PG0	311.5±24.8b	128.6±5.7a	29.2±5.2b	469.6±20.7b
PG5	322.1±28.7b	128.0±5.9a	50.2±9.9a	510.3±33.6b
PG10	325.2±20.3b	132.8±6.6a	56.0±3.6a	514.0±22.6b
PG20	356.9±06.0ab	124.0±8.4a	56.4±3.9a	537.4±10.9ab
PG40	414.5±26.0a	115.2±5.4a	65.6±6.1a	595.3±30.0a
LSD 0.05	68.49	NS	18.65	74.98
Total P (mg/pot)				
PG0	25.8±2.9b	11.1±0.38a	2.5±0.27b	39.3±2.8b
PG5	31.6±2.4b	9.9±0.37a	4.2±0.82ab	45.0±1.9b
PG10	29.5±0.9b	9.8±0.75a	4.8±0.54a	44.1±1.8b
PG20	37.5±3.8a	11.6±1.3a	5.1±0.2a	54.2±4.8a
PG40	42.5±2.5a	10.6±1.1a	5.5±0.81a	58.7±2.9a
LSD 0.05	8.19	NS	1.78	9.76

Means followed by the same letters within a column are not significantly different ($P<0.05$); NS: Non-significant

In sorghum, the %N in both shoots and roots was reduced following the addition of PG (PG5, PG10 and PG20). However, no significant difference was observed between the control and the PG40 treatment (Table 3). In contrast to the dry matter yield, N uptake by sorghum was not affected by the PG supply (Table 2).

Table 2
Dry matter yield, N uptake and P uptake in *Sorghum bicolor* as affected by different rates of phosphogypsum (PG)

Treatments	Shoots	Roots	Whole plant
Dry matter yield (g/pot)			
PG0	10.63±0.96c	6.25±1.47b	16.88±2.40b
PG5	19.75±0.48a	13.63±1.20a	33.38±1.54a
PG10	16.13±1.42b	13.37±0.21a	29.50±1.58a
PG20	17.25±1.01ab	11.63±0.32a	28.90±1.60a
PG40	11.38±1.02c	8.30±0.97b	19.71±1.90b
LSD 0.05	3.09	3.04	5.59
Total N (mg/pot)			
PG0	240.4±15.4a	68.9±11.7b	309.2±24.9a
PG5	225.0±5.3a	104.7±11.8a	329.7±10.9a
PG10	216.4±22.6a	104.5±4.9a	321.0±27.2a
PG20	231.0±12.3a	80.1±3.9ab	311.2±13.2a
PG40	240.5±16.5a	85.4±4.2ab	325.9±18.9a
LSD 0.05	NS	24.6	NS
Total P (mg/pot)			
PG0	14.1±1.2c	6.9±1.6c	21.1±2.7c
PG5	23.2±0.8a	12.1±0.2a	35.3±0.9a
PG10	21.4±1.7ab	14.2±0.8a	35.5±2.5a
PG20	22.0±2.6a	9.1±0.8bc	31.2±3.3ab
PG40	16.6±1.5bc	8.4±1.2bc	24.9±1.7bc
LSD 0.05	5.06	2.78	7.14

Means followed by the same letters within a column are not significantly different (P<0.05); NS: Non-significant

Table 3
Concentrations of nitrogen and phosphorus in different plant parts of *Sesbania aculeata* and *Sorghum bicolor* as affected by different rates of phosphogypsum (PG)

Treatments	Shoots		Roots		Nodules	
	%N	P (μ g/g)	%N	P (μ g/g)	%N	P (μ g/g)
Sesbania						
PG0	2.15±0.01a	1751±60b	1.29±0.05a	1114±48.9a	4.48±0.52b	3807±41.5b
PG5	2.09±0.19a	1980±194ab	1.23±0.04a	961±27.8ab	6.07±0.26a	5081±123.3a
PG10	1.97±0.07a	1795±66b	1.22±0.02a	905±51.9b	5.76±0.45a	4854±62.1a
PG20	2.09±0.09a	2171±179a	1.21±0.07a	1130±113a	5.60±0.04a	5111±317.9a
PG40	2.16±0.13a	2213±275a	1.18±0.02a	1084±73.5a	5.77±0.33a	4567±253.0a
LSD 0.05	NS	579.5	NS	208.9	1.09	581.2
Sorghum						
PG0	2.28±0.07a	1327±16.2ab	1.15±0.01a	1138±82.7a	–	–
PG5	1.14±0.03b	1179±54.3b	0.79±0.14b	903±73.6bc	–	–
PG10	1.34±0.07b	1328±27.6ab	0.78±0.02b	1060±51.5ab	–	–
PG20	1.35±0.08b	1267±84.2b	0.69±0.01b	779±30.6c	–	–
PG40	2.13±0.10a	1460±67.0a	1.06±0.08a	1037±80.6ab	–	–
LSD 0.05	0.22	168	0.25	201.5	–	–

Means followed by the same letters within a column are not significantly different (P<0.05); NS = Non-significant

Effect of PG on phosphorus content and uptake

The phosphorus concentration (μg/g) in sesbania shoots was significantly higher in the PG20 and PG40 treatments than in the control (Table 3). However, the effect of PG was more pronounced on sesbania nodules than on the other plant parts, a significant increase in P concentration being observed in all the PG treatments (Table 3). The P uptake (mg/pot) in sesbania plants as affected by PG is shown in Table 1. The amounts of P accumulated in the plants increased by 45 and 65% in the shoots, and by 38 and 49% in the whole plant in the PG20 and PG40 treatments, respectively. However, the roots were apparently not affected by PG application. In the nodules, the P uptake significantly increased with increasing rates of PG application, with average percentage increments of 68, 92, 104 and 120% for the PG5, PG10, PG20 and PG40 treatments, respectively. These values were relatively similar to those of nodule N uptake data.

The P uptake and concentration in sorghum plants as affected by PG are shown in Tables 2 and 3, respectively. No significant difference in shoot P concentration was obtained between the control and the PG treatments. In sorghum roots, the PG5 and PG20 treatments had significantly lower values of P concentration than that of the control (Table 3). However, the amounts of P accumulated in the shoots (Table 2) were significantly higher for the PG5, PG10 and PG20 treatments than in the control, though no significant difference was found between PG40 and the control. The percentage increments of P uptake caused by the PG amendment in the whole plant of sorghum were estimated as 67, 68 and 49% for the PG5, PG10 and PG20 treatments, respectively.

Effect of PG on N uptake from available sources

Shoots of *Sesbania aculeata* treated with PG had lower values of ^{15}N compared to the control receiving no PG (Table 4). This indicates that a dilution effect had taken place, and that a portion of the sesbania N was derived from the N_2 fixation process, which was considerably enhanced due to the PG supply. The percentage of N derived from the atmosphere (%Ndfa) in sesbania shoots was 43.8, 56.4, 51.3 and 45.5% in the PG5, PG10, PG20 and PG40 treatments, respectively whereas it was only 15.3% in the control (Table 5). In the root system (roots + nodules), the percentages of Ndfa were relatively higher than those in the shoots (80, 84, 83, 86 and 75%, for the aforementioned treatments, respectively). This was probably due to the presence of the nodules, which had a lower ^{15}N value than the shoots. Estimates of total %Ndfa were 57, 67, 62 and 58% in the PG5, PG10, PG20 and PG40 treatments, respectively, but only 35% in the control (PG0) (Fig. 1).

As regards the N derived from fertilizer (Ndff), the addition of PG resulted in lower values of %Ndff compared to the control, particularly in the shoots. However, the values of Ndff seemed to be unaffected by the PG application rates. Moreover, adding PG decreased both the percentage and the amount of N derived from the soil (Ndfs) in sesbania plants (Table 5).

Table 4
Percentages of ^{15}N atom excess in different plant parts of *Sesbania aculeata* and *Sorghum bicolor* as affected by different rates of phosphogypsum (PG)

Treatment	Shoots	Roots	Nodules
Sesbania			
PG0	0.9005±0.028a	0.813±0.068a	0.212±0.026a
PG5	0.7760±0.058ab	0.864±0.039a	0.191±0.012ab
PG10	0.6690±0.094b	0.735±0.098a	0.160±0.036ab
PG20	0.6723±0.032b	0.824±0.033a	0.175±0.017ab
PG40	0.6022±0.054b	0.730±0.045a	0.147±0.009b
LSD 0.05	0.196	NS	0.065
Sorghum			
PG0	1.063±0.024c	0.0940±0.03c	–
PG5	1.381±0.038b	1.211±0.013ab	–
PG10	1.536±0.035a	1.334±0.053a	–
PG20	1.381±0.067b	1.268±0.041a	–
PG40	1.106±0.046c	1.105±0.055b	–
LSD 0.05	0.134	0.125	–

Means followed by the same letters within a column are not significantly different ($P<0.05$); NS = Non-significant

Table 5
Percentages and amounts of nitrogen derived from fertilizer (Ndff), soil (Ndfs) and atmosphere (Ndfa) in shoots and root system of *Sesbania aculeata* as affected by different rates of phosphogypsum (PG)

Treatment	Ndff		Ndfs		Ndfa	
	Shoots	Root system	Shoots	Root system	Shoots	Root system
%						
PG0	18.01±0.8a	4.68±0.3a	66.74±2.1a	20.23±1.2a	15.25±2.7b	75.09±1.5c
PG5	15.52±1.7ab	4.76±0.5a	40.68±4.5b	14.90±1.5b	43.80±6.2a	80.34±2.0bc
PG10	13.38±1.9b	4.23±0.8ab	30.18±4.3c	11.62±2.1b	56.44±6.2a	84.16±2.9ab
PG20	13.45±0.7b	4.14±0.1ab	35.22±1.7bc	12.17±0.3b	51.33±3.3a	83.69±0.4ab
PG40	12.05±1.1b	3.16±0.1b	42.41±3.8b	11.13±0.7b	45.50±4.9a	85.70±0.9a
LSD 0.05	3.91	1.31	10.46	3.98	14.33	5.29
mg N/pot						
PG0	56.5±6.1a	7.4±0.5ab	209.3±22.8a	31.8±1.9a	45.8±06.3b	118.6±8.3b
PG5	50.5±4.4a	8.6±1.3a	132.3±11.6bc	26.9±4.3ab	149.4±34.0a	142.7±9.6ab
PG10	43.1±5.8a	7.9±1.3ab	97.1±13.3c	21.8±3.7b	185.0±27.1a	159.0±10.6a
PG20	48.0±2.3a	7.5±0.5ab	125.7±06.0c	21.9±1.3b	183.3±09.4a	151.0±07.9a
PG40	49.7±4.8a	5.7±0.3b	174.8±17.1ab	20.0±0.8b	190.0±27.3a	155.1±05.4a
LSD 0.05	NS	2.7	45.86	8.31	70.89	25.86

Means followed by the same letters within a column are not significantly different ($P<0.05$); NS = Non-significant

In contrast to *Sesbania aculeata*, sorghum plants treated with PG had a higher ^{15}N atom excess value than the control (Table 4), indicating the beneficial effect of PG in enhancing fertilizer N uptake.

The percentages and amounts of Ndff in the whole plants of sorghum significantly increased compared with the control, at increasing rates of PG, except for the PG40 treatment, where the difference was not significant (Table 6). On the other hand, while the %Ndfs significantly decreased in the PG5, PG10 and PG20 treatments as compared to the control, PG application had no significant effect on the total amount of Ndfs in the whole sorghum plant (Table 6).

Effect of PG on fluoride (F^-) content

The concentrations of fluoride (F^-) in the shoots ranged from 4.3 to 5.6 mg/kg for sesbania and from 6.4 to 9.9 mg/kg for sorghum, while the F^- concentrations in the roots and nodules were 26–34 mg/kg and 11–22 mg/kg, respectively, for sesbania (Fig. 2). However, the sorghum roots had a high F^- concentration even in the control treatment (PG0).

Discussion

Adding PG can be considered an effective way of improving soil properties and increasing plant productivity (Al-Oudat et al., 1998; 2004; Al-Karaki and Al-Omoush, 2002). The present study showed that the dry matter content of sorghum was doubled when the soil was supplied with the lowest rate of PG (5 t/ha). In sesbania, however, only the highest rate of PG (40 t/ha) was found to have a significant effect on the dry matter yield. This indicates that plant species differ greatly in their response to the rate of PG application. Moreover, the beneficial effect of PG on the dry matter yield was evident in the shoots and root systems of both plant species. The main beneficial effect of PG on the root system of sesbania was restricted only to its nodules.

Table 6
Percentages and amounts of nitrogen derived from fertilizer (Ndff) and soil (Ndfs) in different plant parts of *Sorghum bicolor* as affected by different rates of phosphogypsum (PG)

Treatment	Ndff			Ndfs		
	Shoots	Root system	Whole plant	Shoots	Root system	Whole plant
%						
PG0	21.3±0.48c	18.8±0.61c	20.7±0.4b	78.8±0.48a	81.2±0.61a	79.3±0.42a
PG5	27.6±0.75b	24.2±0.26ab	26.6±0.6a	72.4±0.75b	75.8±0.26bc	73.4±0.61b
PG10	30.7±0.70a	26.7±1.06a	29.4±0.5a	69.3±0.70c	73.32±1.10c	70.6±0.47c
PG20	27.6±1.35b	25.4±0.82a	27.0±1.2a	72.4±1.30b	74.6±0.82c	72.9±1.20bc
PG40	22.1±0.93c	22.1±1.09b	22.1±0.9b	77.9±0.9a	77.9±1.10b	77.9±0.90a
LSD 0.05	2.68	2.50	2.39	2.68	2.50	2.39
mg N/pot						
PG0	51.1±3.7b	13.0±2.3d	64.1±5.3c	189.2±11.9a	55.9±9.4c	245.2±19.7a
PG5	62.2±2.7ab	25.3±2.8ab	87.5±2.1ab	162.8±3.3ab	79.4±9.0ab	242.1±9.5a
PG10	66.6±7.5a	28.0±2.3a	94.6±9.3a	149.8±15.3b	76.5±2.7a	226.3±17.9a
PG20	63.7±4.3b	20.2±0.4bc	83.9±4.1ab	167.3±9.8ab	59.9±3.6bc	227.2±11.5a
PG40	53.3±4.6ab	18.8±0.8cd	72.0±4.9bc	187.2±12.8a	66.6±4.0abc	253.8±15.6a
LSD 0.05	14.49	5.90	17.09	34.3	19.37	NS

Means followed by the same letters within a column are not significantly different ($P<0.05$); NS = Non-significant

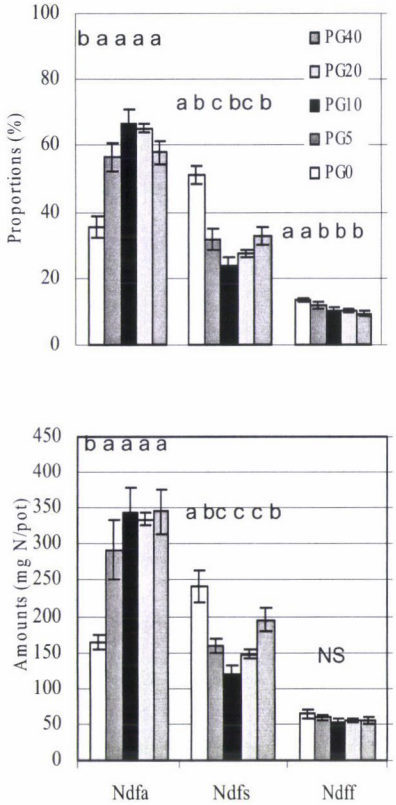

Fig. 1. Proportions and amounts of nitrogen derived from the atmosphere (Ndfa), soil (Ndfs) and fertilizer (Ndff) in the whole plant of *Sesbania aculeata* as affected by different rates of phosphogypsum (PG)

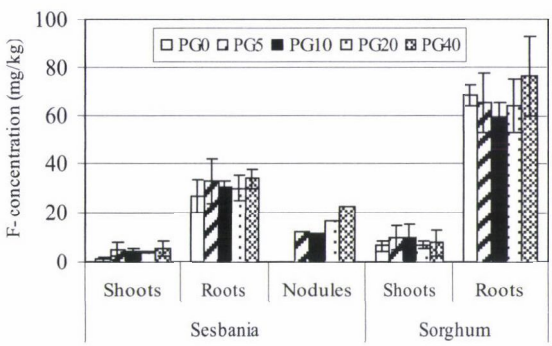

Fig. 2. Fluoride concentration (mg/kg) in different plant parts of *Sesbania aculeata* and *Sorghum bicolor* as affected by different rates of phosphogypsum (PG)

Although the dry matter yield of sorghum significantly increased with additional PG supplements, the shoot N yield was not affected. This was because the %N in sorghum shoots supplied with PG (at the PG5, 10 and 20 rates) was significantly lower than in the control (PG0). In this context, several authors reported that the nitrogen concentration (%N) declined as a function of aerial biomass accumulation (Plenet and Lemaire, 1999). The beneficial effect of adding PG to the soil on sorghum N yield was restricted to the roots, particularly at the PG5 and PG10 rates. On the other hand, PG had a beneficial effect on the P accumulation in the shoots and roots of sorghum, particularly when one of the latter rates was used. Therefore, it can be concluded that adding 5 t/ha of PG to the soil specified in this study was sufficient to obtain optimal biomass of sorghum with high phosphorus content.

The beneficial effect of PG application on *Sesbania aculeata* was more pronounced in terms of nodulation and N_2 fixation than on host plant growth. The nodule mass increased with increasing PG rates. The supply of 10 t/ha PG appeared to be sufficient for optimal nodulation. Increasing PG beyond this rate did not lead to additional increments in nodule mass. In addition, the study demonstrated that PG has a specific role in nodule functioning in *Sesbania aculeata*. The highest value of $\%N_2$ fixation (67%) was obtained following the addition of 10 t of PG/ha, whereas it was only 35% for the control treatment (PG0). Furthermore, the amounts of N_2 fixed doubled when the soil was supplied with PG, particularly when the PG10 rate was applied (Fig. 1). Moreover, the enhancement of N_2 fixation in sesbania in response to PG supplies was associated with a decrease in soil N uptake. This result has important implications for agricultural practice, helping to maintain soil N when sesbania is used in rotation systems with cereals.

Several nutrients essential for the growth of plants or bacteria play a specific role in nodulation and/or N_2 fixation, including calcium (Ca), sulphur (S), zinc (Zn), silicon (Si), copper (Cu), iron (Fe), magnesium (Mg), phosphorus (P) and others (Giller and Wilson, 1993). Deficiencies in these or other nutrients essential for the growth of bacteria or plants may cause reductions in nodulation and N_2 fixation.

PG is composed primarily of Ca, S, P and Si, while small quantities of other nutrients (Fe, Mg and Zn) are also present. In India, phosphogypsum serves as an important source of S and Ca for chickpea growth (Ghosh and Sarkas, 2000). In Jordan, Al-Karaki and Al-Omoush (2002) reported that PG could be used as a crude phosphoric fertilizer for direct application to alkaline soils. Al-Masri et al. (2004) reported that Syrian PG contains concentrations of trace elements (e.g. Cu and Zn) comparable to those reported worldwide.

The enhancement of N_2 fixation in sesbania could be mainly attributed to an improvement in P availability after the application of PG to the soil. Khalil et al. (1990) reported that high application rates of PG on calcareous soil significantly increased the available soil P. The initial P concentration in the soil

used in the present study was relatively low (3.7 μg/g), so P has to be added to provide sufficient P for the growth of both plant species, particularly the legume, which requires more P for the optimal functioning of the nodules (Tang et al., 2001; Israel, 1987). The addition of poorly soluble forms of P such as PG to the soil, which often have low concentrations of available P, may ensure the continuous solubilization of PG to provide an adequate amount of P for plant growth (Al-Karaki and Al-Omoush, 2002), including the nodules. The increase in N_2 fixation by PG application was associated with increases in the N and P concentration and their accumulation in the nodules. Higher P concentrations were observed in the nodules than in roots and shoots when PG was applied. This could indicate that the nodules are a strong sink for P.

Crop yields and the quality of a variety of fruit, vegetable, grain, forage and oil seeds have been found to be higher on PG-amended soils (Rutherford et al., 1994). The wide-scale use of PG is restricted by several concerns, including its content of natural radionuclides, heavy metals and fluoride. The U.S. E.P.A. ruled that PG could only be permitted for use in agriculture if the average activity of ^{226}Ra in the PG did not exceed 370 Bq/kg (Rutherford et al., 1994). The present study showed that the specific activity of ^{226}Ra in the PG applied (245 Bq/kg) did not exceed this. Because of the low radionuclide concentration in the PG used, and the small number of plant samples, the radioactivity and heavy metals in the plant tissues were not determined in this study. In this context, it is worth mentioning that the use of Syrian PG, with 350 and 400 Bq/kg of radioactivity (Othman and Mahroka, 1994), increased the productivity of clover and barley without increasing the radioactivity of the barley grains or of the vegetative parts of both plants (Al-Oudat et al., 1998). The latter study examined the effects of mixing PG with different types of soils (up to 25%) to mimic the effects of adding 5 t/ha to local soils for 100 consecutive years. The radioactivity of plants grown in these mixtures were below the analytical detection limits (1.1 Bq/kg dry matter) in all the treatments. Furthermore, Al-Oudat et al. (2004) reported that the radioactivity in shoots of *Kochia scoparia* grown on a soil–PG mixture were below the detection level. Mays and Mortved (1986) reported that PG may be applied to agricultural soils at relatively high disposal rates without increasing the levels of Cd or radioactivity in maize, wheat or soybean grains. In addition, Alcordo and Rechcigl (1993) concluded that the concentrations of radionuclides, heavy metal impurities and other pollutants found in PG did not appear to constitute environmental hazards to the groundwater, soil, crop tissue and ambient atmosphere at the rates normally used in agriculture.

Regarding the fluoride (F^-) content in different plant parts of sesbania and sorghum, the results showed that the concentrations of F^- in the shoots of both plants were lower than 10 mg/kg, including the control treatment (PG0). This value did not exceed the acceptable level reported by the Ontario Ministry of the Environment of Canada (OME), which established that the upper limit of the

normal background concentration of F^- in the foliage was 35 mg/kg (Rutherford et al., 1994). Similarly, Al-Oudat et al. (2004) reported that the fluoride concentration in the foliage of *Kochia scoparia* grown on soil amended with phosphogypsum remained below the acceptable level.

The concentrations of F^- in sesbania roots (26–34 mg/kg) and nodules (11–22 mg/kg) were less than 35 mg/kg. However, sorghum roots had a high F^- concentration even in the control treatment (PG0). These results indicate that the initial F^- concentration was relatively high in the soil used in the present study, and that the addition of PG did not significantly increase the F^- concentrations in plant tissues. On the other hand, the roots seemed to be a strong sink for F^-, particularly in sorghum, suggesting that plant species and plant parts differ in their response to fluoride concentration. These results should be considered when PG is used as an amendment to agricultural soil, particularly in root crops.

Conclusions

This study has provided valuable new information on the impact of applying phosphogypsum on N_2 fixation using the ^{15}N isotope dilution method. The data of the present study showed that:

1. Supplying soil with PG, particularly at a rate of 10 t/ha, can be considered as a useful agricultural practice for improving nodulation and N_2 fixation in *Sesbania aculeata* with minimal soil N uptake. This is probably because PG is a source of P and other nutrients that are essential for the N_2 fixation process.
2. The beneficial effect of PG was less pronounced in sesbania than in sorghum, where the dry matter yield doubled when the soil was supplied with the lowest rate of PG (5 t/ha).
3. The fluoride (F^-) content in the shoots of sesbania and sorghum did not exceed the critical level.

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OPTIMUM TIME FOR PHOSPHORUS FERTILIZATION ON EGYPTIAN ALLUVIAL SOIL

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The rapid fixation of phosphorus fertilizers in soil with a high content of calcium carbonate requires efficient management methods for phosphorus fertilization, especially as regards the time of application. For this purpose, a field experiment was carried out in the summer season of 2006/2007 in the experimental farm of the Soils Department, Faculty of Agriculture, Mansoura University, Egypt to evaluate the best time for phosphorus fertilization to cowpea on Egyptian alluvial soil. The results showed that adding half the recommended phosphorus fertilization rate at sowing and the other half before the first irrigation was the best treatment to enhance phosphorus fertilizer use efficiency, contributing to an increased uptake of phosphorus by cowpea, reflected in the higher phosphorus concentration in the grains. Increased phosphorus uptake also caused an increase in the nitrogen and potassium concentrations in cowpea grains, whereas the zinc and iron concentrations decreased.

Key words: Egyptian alluvial soil, phosphorus fertilization, application date

Introduction

Phosphorus is one of the 17 essential elements required for plant growth and development, and one of the three most important nutrients, with nitrogen and potassium. Phosphorus contributes to many vital functions in the plant, such as early root and seedling growth, improved winter hardiness, promotion of early heading and uniform maturity, seed formation and quality, and increased water use efficiency. While these effects are more visible, phosphorus also plays a number of unseen roles such as in photosynthesis, energy storage and transfer, respiration and cell division. Crops deficient in phosphorus tend to develop slower, exhibit limited growth potential, and yield less than expected (Johnston, 2001). This is attributed to the low availability of soil phosphorus, especially in arid and semi-arid regions, caused by intense calcium phosphate fixation (Diez

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et al., 1992). Egyptian farmers add the whole amount of phosphorus fertilizer at the soil ploughing stage. Accordingly, most of the phosphorus content is fixed in an insoluble phase unavailable for plant uptake, leading to decreasing phosphorus fertilizer use efficiency. Phosphorus deficiency in legumes has been shown to decrease the whole plant leaf area and it is clear that leaf area development is an important factor in crop production (Israel and Rufty, 1988; Fredeen et al., 1989; Qiu and Israel, 1994). It also has a vital function in the photosynthesis process, which utilizes light energy in the presence of chlorophyll to combine carbon dioxide and water into simple sugars, with the energy being captured in adenosine triphosphate (ATP). ATP is then available as an energy source for many other reactions that occur within the plant, and sugars are used as building blocks to produce other cell structural and storage components.

The nitrate metabolism is also related with the phosphorus supply, as the absorption and reduction of nitrate is an energy-consuming process, and the energy is supplied by ATP, which contains phosphorus. Consequently it has a major role in protein content (Wang and Li, 2004). The objective of this study was to examine the best time for phosphorus application in order to increase phosphorus use efficiency, cowpea grain yield and nutrient concentration in the grains.

Materials and methods

Field experiments were carried out at the experimental farm of the Faculty of Agriculture, Mansoura University, Egypt (31°04'N, 31°35'E, 7 masl) to investigate the best time for phosphorus fertilization to cowpea (*Vigna sinensis*) grown on Egyptian alluvial soils.

Surface soil samples (0–30 cm) collected from the examined field were air-dried, crushed, passed through a 2 mm sieve and preserved for analysis. The physical and chemical characteristics of the soil, listed in Table 1, were determined as follows: mechanical analysis using the pipette method (Dewis and Fertias, 1970), saturation percentage and field capacity using the methods described by Richards (1954), bulk density using the paraffin wax method (Dewis and Fertias, 1970), organic matter content with the Walkley and Black method, described by Hesse (1971), total carbonate gasometrically using a Collins Calcimeter and calculated as calcium carbonate according to Dewis and Fertias (1970), soil reaction (pH) from a saturated soil paste using a combined electrode pH meter (Richards, 1954), total soluble salts (dS m^{-1}) by measuring the electrical conductivity of a saturated soil paste extract (Jackson, 1967), amounts of water-soluble cations (Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}) and anions (CO_3^{2-}, HCO_3^{-} and Cl^{-}) in saturated soil paste extract (Hesse, 1971), and sulphate (SO_4^{2-}) as the difference between total cations and anions.

The available nitrogen was extracted using 2.0 M KCl and determined with the macro-Kjeldahl method (Hesse, 1971), available phosphorus was extracted with 0.5 M $NaHCO_3$, pH 8.5, and determined colorometrically at a wavelength of 660 nm after treatment with ammonium molybdate and stannous chloride (Jackson, 1967), and available potassium was determined by extraction with 1.0 M ammonium acetate, pH 7.0, using a flame photometer (Hesse, 1971).

The experimental design was a complete randomized block with three replicates, and the following phosphorus treatments were applied: A. Control treatment (without phosphorus fertilization); B. Whole dose at ploughing; C. Whole dose at sowing; D. Whole dose before the first irrigation (with thinning); E. Whole dose before the second irrigation; F. Half dose at ploughing, other half at sowing; G. Half dose at sowing, other half before the first irrigation; H. Half dose before the first irrigation, other half before the second irrigation; I. Half dose before the second irrigation, other half before the third irrigation.

Table 1
Physical and chemical characteristics of the experimental soil

Soil properties		Values
Physical properties		
Particle size distribution	Sand (%)	23.30
	Silt (%)	25.50
	Clay (%)	50.20
	Soil texture	Clayey
Hygroscopic water (%)		7.36
Saturation percentage (%)		68
Field capacity (%)		34
Organic matter (%)		1.82
Bulk density (g cm^{-3})		1.31
Chemical properties		
Calcium carbonate (%)		4.18
*pH (Soil paste)		7.6
**EC (dS m^{-1})		1.56
Soluble cations (meq L^{-1})	Ca^{2+}	5.79
	Mg^{2+}	2.39
	Na^{+}	6.84
	K^{+}	0.28
Soluble anions (meq L^{-1})	CO_3^{2-}	0
	HCO_3^{-}	5.13
	Cl^{-}	6.89
	SO_4^{2-}	3.28
Available nutrients (mg kg^{-1})	Nitrogen	37.35
	Phosphorus	11.21
	Potassium	321

*Determined in soil paste; **Determined in soil paste extract

Before cultivation, the soil was fertilized with 50 m^3 ha^{-1} farmyard manure, then ploughed and left two weeks for solarization. The phosphorus fertilization rate was 500 kg ha^{-1} calcium superphosphate (16% P_2O_5), applied as described above. The crop was grown using traditional management methods.

At the harvest stage, plants were randomly chosen from each replicate, oven dried at 70°C, ground using stainless steel equipment and preserved for analysis. To determine nutrient concentrations in plant tissues, 0.2 g from each ground oven-dried sample was digested with 5 cm^3 of a 1:1 mixture of H_2SO_4 and $HClO_4$, as described by Peterburgski (1968). Nitrogen was determined by the micro-Kjeldahl method (Hesse, 1971), phosphorus colorimetrically at 680 nm using a spectrophotometer (Spekol) (Jackson, 1967), and potassium using a Gallenkamp flame photometer (Jackson, 1967).

The data were statistically analysed and differences between treatment means were compared using the least significant differences (LSD) method at the 5% level using CoStat (1998–2004) software.

Results and discussion

Effect of phosphorus fertilization time on grain yield

Phosphorus fertilization treatments had a highly significant effect on cowpea grain yield, the best results being recorded for Treatment G, followed by Treatment F. It could thus be seen that partitioning the fertilization dose improved the grain yield, whereas delaying the fertilization doses in Treatments H and I decreased the grain yield. This could be mainly attributed to the fact that phosphorus is an essential element in the early growth stages (Segars and Usherwood, 1997; Slaton et al., 2002). It was also clear that adding the whole phosphorus fertilization rate at sowing was better than adding it at ploughing, as this resulted in a reduction in phosphorus fixation.

Effect of phosphorus fertilization time on phosphorus concentration in cowpea grains

The results in Table 2 indicated that phosphorus fertilization treatments had a highly significant effect on the phosphorus concentration in the grains. It was clear from the data in Table 3 that Treatment G was the best treatment for increasing phosphorus fertilization use efficiency, proving that dividing the fertilizer into two equal doses was better than adding the whole quantity at once. Similar results were reported by Diez et al. (1992). On the other hand, it was seen that delaying phosphorus fertilization after the early growth stages tended to decrease phosphorus uptake by the plant, which was reflected in the decreasing phosphorus concentration in the grains.

Table 2
Effect of phosphorus fertilization treatments on grain yield (kg ha^{-1}) and nutrient concentration in cowpea

Treatments	Grain yield	P (%)	N (%)	K (%)	Fe (mg kg^{-1})	Zn (mg kg^{-1})
A	6162^{g}	0.18^{h}	2.8^{f}	1.10^{e}	78^{a}	47^{a}
B	10952^{d}	0.38^{d}	3.7^{cd}	2.00^{bcd}	56^{c}	35.6^{b}
C	11986^{c}	0.40^{c}	3.9^{c}	2.10^{bc}	49^{d}	33.3^{cd}
D	9937^{e}	0.37^{d}	3.5^{de}	1.80^{d}	55^{c}	31.6^{de}
E	8547^{f}	0.33^{f}	3.4^{de}	1.86^{cd}	59^{bc}	30^{e}
F	13611^{b}	0.44^{b}	4.2^{b}	2.20^{ab}	45^{de}	34^{bc}
G	15275^{a}	0.46^{a}	4.5^{a}	2.40^{a}	42^{de}	32.6^{cd}
H	8996^{f}	0.35^{e}	3.3^{e}	1.83^{d}	57^{bc}	32^{d}
I	8439^{f}	0.31^{g}	3.2^{e}	1.80^{d}	61^{b}	31.6^{de}
F-test	**	**	**	**	**	**
LSD at 0.05	854	0.02	0.29	0.19	4.22	1.76

A. Without phosphorus fertilization, B. Whole dose at ploughing, C. Whole dose at sowing, D. Whole dose before 1st irrigation (with thinning), E. Whole dose before 2nd irrigation, F. Half dose at ploughing, other half at sowing, G. Half dose at sowing, other half before 1st irrigation, H. Half dose before 1st irrigation, other half before 2nd irrigation, I. Half dose before 2nd irrigation, other half before 3rd irrigation; Mean values followed by the same letter within treatments are not significantly different ($p < 0.05$) according to the LSD test.

Table 3
Effect of phosphorus fertilization treatments on phosphorus uptake by grains and phosphorus fertilizer use efficiency

Treatments*	Phosphorus uptake (kg ha^{-1})	Phosphorus fertilizer use efficiency (%)
A	11.0916	–
B	41.6176	6.10
C	47.9440	7.37
D	36.7669	5.14
E	28.2051	3.42
F	59.8884	9.76
G	70.2650	11.83
H	31.4860	4.08
I	26.1609	3.01

*For treatments see Table 2

Effect of phosphorus fertilization time on nitrogen concentration in cowpea grains

The data presented in Table 2 revealed that the phosphorus fertilization time had a highly significant effect on the nitrogen concentration in the grains, as also reported by Becker et al. (1991) and Engels et al. (1995). It is well known that a sufficiency of available phosphorus in the rhizosphere encourages root growth and root hair formation in the early growth stages, thus increasing nitrogen uptake. A positive interaction has also been reported between the amount of phosphorus available for growth and the strength of the symbiotic process in nodulated crop legumes (Sa and Israel 1991; 1995; Engels et al., 1995; Materon and Ryan, 1995; Elabbadi et al., 1996; Leidi and Rodriguez-Navarro, 2000). Moreover, phosphorus plays a major role in nodule formation, influencing the number and fresh weight of nodules (El-Keiy and El-Sayed, 1991; Wan Othman et al., 1991; Crews, 1993). The results revealed that Treatment G was ranked first, followed by Treatment F, which could be attributed to the splitting of the fertilizer rate, offering adequate amounts of available phosphorus in the early, more physiologically active growth stages (Segars and Usherwood, 1997).

Nevertheless, splitting the phosphorus fertilizer dose in Treatments H and I did not achieve the same positive effect as the previous treatments, because of the lower quantity of available phosphorus in the early growth stages. It is clear from Figure 1, that there is a positive correlation between the phosphorus and nitrogen concentrations in cowpea grain, indicating that increasing phosphorus uptake enhanced the nitrogen concentration in the grains. The Pearson correlation factor was 0.9157 and the regression equation:

$$Y = 0.545 \text{ Ln } (x) - 0.3363$$

where:

y = Nitrogen concentration (%) in cowpea grains,
x = Phosphorus concentration (%) in cowpea grains.

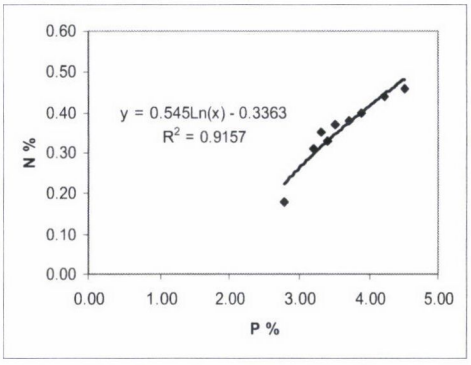

Fig. 1. Relationship between phosphorus and nitrogen concentrations in cowpea grains

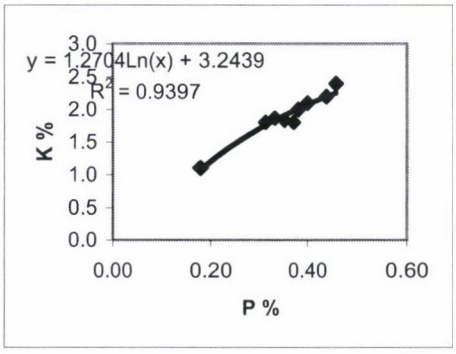

Fig. 2. Relationship between phosphorus and potassium concentrations in cowpea grains

Effect of phosphorus fertilization time on potassium concentration in cowpea grains

The data in Table 2 show that the phosphorus application time had a highly significant effect on the potassium concentration in cowpea grains, and it is clear that dividing the fertilization dose increased the potassium concentration. Similar results were also obtained by El-Hamdi (1990) and Xu et al. (2002). This effect could be attributed to the function of phosphorus in improving root growth, leading to the exploration of greater soil volume and increased potassium uptake. Moreover, it is well known that there is a synergistic relationship between the calcium concentration in the soil (which is applied with phosphorus in the form of calcium superphosphate) and potassium uptake by plants (Marchner, 1995), which could also be associated with the increasing potassium concentration in the grains. A positive correlation was revealed between the phosphorus and potassium concentrations in the grains (Fig. 2), with a Pearson correlation factor of 0.9397 and the regression equation:

$$y = 1.2704 \text{ Ln } (x) + 3.2439$$

where:

y = Potassium concentration (%) in cowpea grains,
x = Phosphorus concentration (%) in cowpea grains.

Effect of phosphorus fertilization time on zinc concentration (mg kg^{-1}) in cowpea grains

It is clear from Table 2 that the later application of phosphorus caused a highly significant reduction in the zinc concentration in the grains, due to the antagonistic relationship between phosphorus and zinc uptake by plants (Payne et al., 1986).

A negative relationship was found between the phosphorus and zinc concentrations in cowpea grains (Fig. 3), with a Pearson correlation factor (R^2) of 0.5827 and the regression equation:

$$y = -13.923 \text{ Ln } (x) + 19.495$$

where:

y = Zinc concentration (mg kg^{-1}) in cowpea grains,

x = Phosphorus concentration (%) in cowpea grains.

Effect of phosphorus fertilization time on iron concentration (mg kg^{-1}) in cowpea grains

Phosphorus fertilization decreased the iron concentration in cowpea grains, as illustrated in Table 2. This was related to the antagonistic effect of phosphorus on iron uptake.

The negative relationship between the phosphorus and iron concentrations in cowpea grains (Fig. 4) had a Pearson correlation factor (R^2) of 0.9546 and the regression equation:

$$y = -36.654 \text{ Ln } (x) + 17.117$$

where:

y = Iron concentration (mg kg^{-1}) in cowpea grains,

x = Phosphorus concentration (%) in cowpea grains.

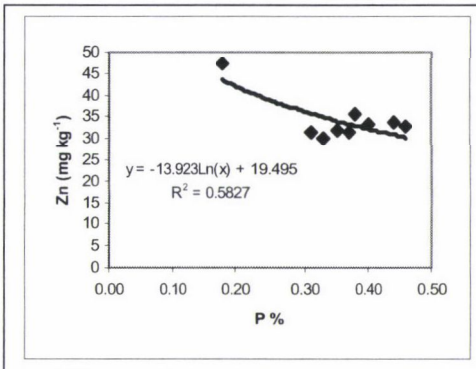

Fig. 3. Relationship between phosphorus and zinc concentrations in cowpea grains

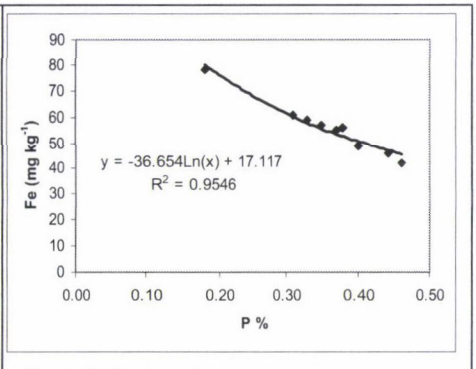

Fig. 4. Relationship between phosphorus and iron concentrations in cowpea grains

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Short communication

TRADITIONAL MAIZE HETEROSIS SOURCES IN EASTERN CENTRAL EUROPE

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The main characteristics of the European heterosis sources Mindszentpusztai, Rumai and Many-Rowed Early Flint probably developed in Eastern Central Europe. Little time and few funds are currently spent on their improvement, so they are constantly being eroded in number and their relative breeding value has declined. The elaboration of methods for the utilisation of European sources could be of great assistance in achieving improvements in maize yield potential and crop safety on a global scale. The first step in this work will be the clarification of the possible origin of the heterosis sources.

Key words: maize, breeding, diversity, genetic resources

Introduction

The long-term success of maize production is based on a constant increase in average yields. This, in turn, is dependent on continual improvements in the production technology and on the introduction of newer hybrids with greater yield potential.

In the opinion of leading scientists, over the last 20 years lack of funds for the breeding of basic material, essential to ensure an improvement in yield potential, has led to a slower increase in yield potential and crop safety.

If the breeding of basic material does not provide a continuous supply of efficient, fresh, intact sources for the development of parental lines, the main source of initial breeding material will be restricted to closely related pedigrees. This will inevitably limit breeding progress.

In Eastern Central Europe heterosis sources previously used to good effect could be used to expand the choice of sources if methods are elaborated to utilise them efficiently. This will require studies on the relationships and origin of these sources of heterosis (Trifunovic, 1978; Palaversic et al., 1979; Németh, 1985; Radovic and Jelovac, 1995; Parlov et al., 2003; Drinic et al., 2007; Jambrovic et al., 2009).

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Materials and methods

Pedigrees were collected for the most important parental lines used in earlier years for hybrid maize breeding in Eastern Central Europe (Hadi, 2004; 2005a; b; Hadi et al., 2004). The history of how varieties in this region were bred was investigated using papers published in Hungarian by the breeders themselves or by their contemporaries almost 100 years ago, and in some cases using personal communications from the original breeders of the varieties.

The main results of these investigations were published by Hadi (2003a; b; 2004; 2005a; b; 2006a; b) and Hadi et al. (2004). The present paper presents a summary of the probable origins of the different varieties.

Results

From the 1960s to the 1990s two important dent sources of heterosis, differing from those used in the American Corn Belt, were utilised in Eastern Central Europe. These were complemented by a cold-tolerant, many-rowed early flint source of heterosis, known in Western Europe as Early European Flint, with properties that were quite different from those of American Northern Flint and which probably evolved in Eastern Central Europe.

The variety Mindszentpusztai Yellow Dent (Fig. 1) was almost certainly derived from Leaming (Hadi et al., 2004), which was introduced into Eastern Central Europe in the 1880–1890s after winning a prize at an American Maize Show. This variety was cultivated by the Pap family in the neighbourhood of Baja, in southern Hungary, for nearly 20 years. In 1917 Endre Pap started breeding it using the pedigree method. From 1933 onwards he developed inbred lines (156, 014, 0118b, 0118a, 01), which were then used as the parents of cultivated hybrids. This source of heterosis was also used in other parts of Europe. After the lines developed by Endre Pap, at least 14 second-cycle lines also became known.

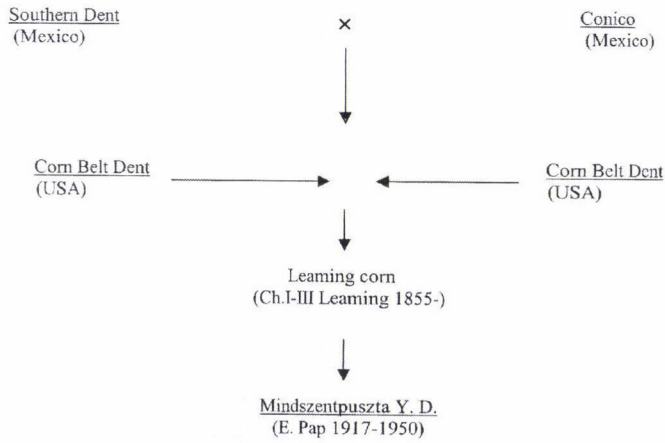

Fig. 1. Mindszentpuszta heterosis source

The Ruma source of heterosis (Fig. 2) originated from "Early Golden" (probably Livingstone's Early Golden), which was introduced into Hungary in the 1890s. In the course of production this probably became mixed with Early Bánát Flint on the farm belonging to the Pejacsevics family, and it was from this population that Rudolf Fleischmann selected over 200 plants in 1908, including that known as No. 122. Fleischmann made use of pedigree selection, adapting the method used in wheat breeding to make it suitable for maize breeding. The lines were all grown in isolation. Many popular varieties can be traced back to line No. 122, including Ruma Golden Y.D., Vukovár Y.D., Bellye Golden Y.D. and finally "F" Golden Y.D., which was later separated into "F" Korai (Early) and "F" Mezőhegyesi on the basis of the flowering date. At least 22 first-cycle and 8 second-cycle lines from these sources were used as parental lines (Hadi, 2005a).

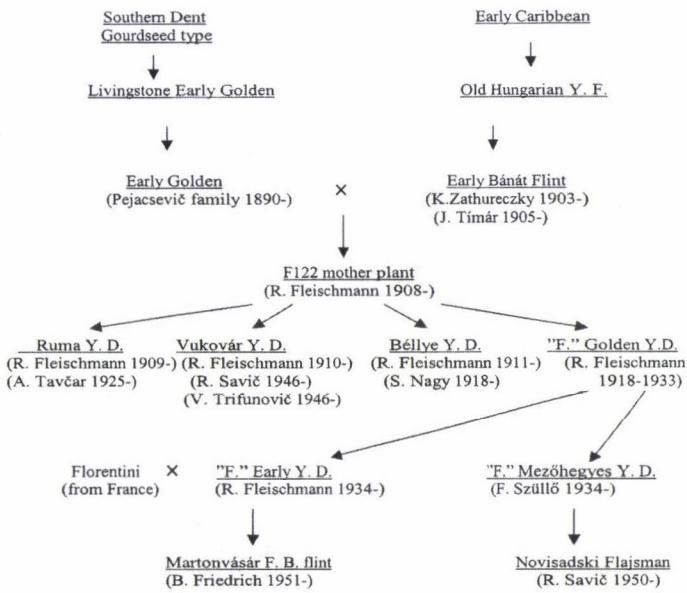

Fig. 2. Ruma heterosis source

The popcorn variety Chutucuno Chico, which was of Andean origin, was introduced into Hungary via Italy, under the name Cinquantino, in the early 1800s for human consumption (as porridge) (Fig. 3). It was crossed, spontaneously or intentionally, with several sources, but particularly with Old Hungarian Yellow, which was of Caribbean origin. Indirect evidence suggests that this source contributed to the development of Early European flint, also known as Alpine Flint (Hadi, 2005b). The common ancestry of the Eastern Central European Multi-rowed Flints and the European Early Flints is suggested

by the phenological similarity observed for many groups of traits. The Eastern Central European Multi-rowed Flints evolved earlier than the European Early Flints and some of the more popular varieties in this group (Legkorábbi Székely, Putyi) were grown for several decades in France, Germany, Bohemia, Poland and Russia (particularly after winning the top award at the World Exhibition in Paris). At least two parental lines from this source were used in Hungary for hybrid development.

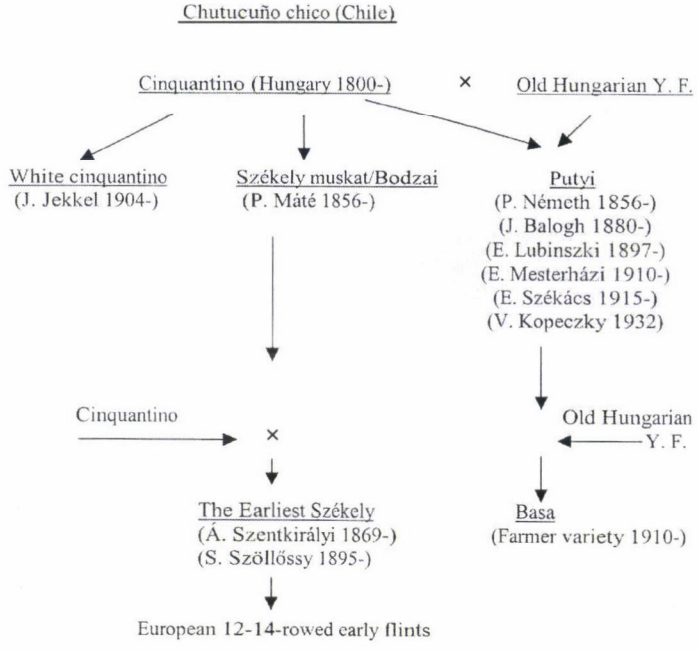

Fig. 3. Many-rowed flint source of heterosis

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Short communication

DIFFERENCES IN STAINING OF THE UNICELLULAR ALGAE *Chlorococcales* AS A FUNCTION OF ALGAENAN CONTENT

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Algal strains belonging to the *Chlorococcales (Chlorophyceae)* show significant differences in the extent of staining with the commonly used dye, crystalline violet. This seems to depend on the cell wall composition and on the occurrence of the acetolysis-resistant biopolymer algaenan in the algal cells.

Eighteen algal strains were investigated using 24 h staining with 0.2% crystalline violet and it was confirmed that algal strains which did not contain algaenan and had a trilaminar structure in the cell wall showed strong staining ability, while non-algaenan strains were stained very weakly, if at all. The simple method presented here may be helpful to distinguish both kinds of algal strains.

Key words: alga, algaenan, *Chlorococcales*, cell wall, crystalline violet, cell staining

Introduction

A correlation has recently been noticed between the penetration of various chemical substances into cells and the ultrastructure of algal cell walls and the occurrence of algaenan (Burczyk, data not published). Previously, Biedlingmeier et al. (1987) showed a connection between the penetration of linear alkylobenzene sulphate (LAS) detergent and its toxicity to algal cells. Similar investigations showed that *Chlorella emersonii* and *Chlorella vulgaris* differed greatly in their sensitivity to Triton X-100 and dodecylbenzene sulphate (Corre et al., 1996). Further examinations confirmed a correlation between high resistance to detergents and the cell wall ultrastructure.

It was found that the occurrence of algaenan, previously mistakenly called sporopollenin, in the cell wall hindered the penetration of various substances into the cells. Algaenan (ARB – acetolysis-resistant biopolymer) is a polyhydrocarbon exhibiting many analogies with sporopollenin: hydrophobicity,

insolubility in polar and non-polar solvents, high resistance to many chemical agents, such as orthophosphoric acid, detergents, alkali hydrolysis, acetolysis, biological agents and high temperature. The occurrence of algaenan in algal cell walls shows a strong correlation with the ultrastructure of the outer cell wall layer. It was affirmed that algal cells which do not form algaenan have a simple one-layer cell wall, while strains capable of synthetizing algaenan have an additional outer trilaminar structure, similar to typical cell membranes (Burczyk, 1982). Algal strains which do not possess the algaenan layer are much more sensitive to many toxic compounds.

In the present work a simple method is proposed based on differences in staining with crystalline violet, allowing a distinction to be made between non-algaenan and algaenan-containing algal strains.

Materials and methods

Eighteen algal strains of *Ankistrodesmus*, *Chlorella* and *Scenedesmus* were investigated, originating from various collections, as shown in Table 1. All of them were axenic and were screened for the presence of algaenan.

Table 1
Percentage of stained algal cells after 24 hours of staining with 0.2% crystalline violet and 1 hour of rinsing

Strains	Collection*	Occurrence of algaenan	% A	% B
Ankistrodesmus brauni 202-7c	G	+	6.8	1.4
Chlorella fusca C.1.1.10 (Cz_1)	Cz	+	0.0	0.0
Chlorella fusca C.1.1.6 (Cz_2)	Cz	–	100.0	100.0
Chlorella fusca 211-11n	G	+	10.0	2.3
Chl. fusca var. *vacuolata* 211-8p	G	+	10.6	19.4
Chl. fusca var. *vacuolata* 211-8b	G	+	14.1	8.4
Chlorella sp. 4	SP	–	100.0	100.0
Chlorella sp. 5	SP	–	100.0	100.0
Chlorella Kessleri K.	G	–	100.0	100.0
Chlorella sp. 3.83	G	–	100.0	98.0
Chlorella sp. 620	JB	+	19.0	24.2
Chlorella sp. Milogradow 113	SP	+	3.2	1.1
Chlorella vulgaris Beijerick 136	SP	–	100.0	100.0
Chlorella saccharophila 211-1a	G	–	100.0	100.0
Chloralla saccharophila 211-9a	G	–	100.0	100.0
Scenedesmus obliquus PG-1	M	+	1.9	2.6
Scenedesmus obliquus 633	JB	+	0.3	5.0
Scenedesmus quadricauda 449	JB	+	10.3	2.8

*Collections: Cz – Prof. F.-C. Czygan, University of Würzburg, G – University of Göttingen, JB – Prof. J. Burczyk, Medical University of Silesia, Laboratory of Biotechnology, Cieszyn; M – University of Marburg, SP – University of St. Petersburg; +: presence of algaenan; –: absence of algaenan; % A: % of stained cells after 24 hours of staining; % B: % of stained cells after 24 hours of staining and 1 hour of rinsing

The algae were cultivated in 1 l Erlenmeyer flasks containing 0.5 l of nutrient medium according to Kessler and Czygan (1970), as modified for microalgal growth by Burczyk (1982). The cultivation was carried out under sterile conditions at 25±1 °C, under illumination by fluorescent lamps (4000 lux) with a 16/8 h light/dark photoperiod. The flasks were stirred manually twice a day and maintained under these conditions for 30 days. Then 1 ml of suspension culture was added to 1 ml of a 0.2% aqueous solution of crystalline violet and mixed. After 24 h staining, the samples were centrifuged (800 g, 7 min) and the pellet was resuspended in distilled water for an hour to remove excess dye. Finally, the centrifuged algal cells were examined under an optical microscope (Optiplot-2 Microscope, Nikon). The percentage of stained cells was evaluated by determining the cell number using a Bürker hemocytometer.

Qualitative assay of algaenan

The assay was carried out according to Burczyk (1987) with modifications. Algal cells from 30-day-old cultures were centrifuged (800 g, 7 min) in 25 cm^3 glass tubes. After washing the pellet with distilled water, the samples were lyophilized and successively extracted with 10% aqueous KOH (5 h, 100°C) and 5% ethanolic KOH (3 h, 75°C) and finally washed with distilled water (10 min, 100°C). Then the samples were cooled, neutralized with 1 M HCl, washed with water and freeze-dried. The lyophilized samples were suspended in 85% orthophosphoric acid at 28°C for 10 days (acetolysis) and occasionally mixed with a stainless steel rod. After acetolysis the probes were filtered through a glass filter G-3 (Schott) and washed first with hot water and then with ethanol and diethyl ether. The presence of an insoluble sediment after acetolysis was considered as a positive result for the presence of algaenan, while a clear solution indicated the absence of algaenan in the cell wall.

Results

The results of staining the algal strains with crystalline violet are presented in Table 1. Generally, strains containing the biopolymer algaenan showed a substantially lower number of stained cells, if any, after a relatively long period of staining, whilst the non-algaenan strains had a staining rate of almost 100%. Moreover, there was no significant difference between the number of stained cells before and after rinsing with water.

Most of the strains containing the acetolysis-resistant biopolymer were characterized by a very low percentage of stained cells, between 0 and 10%. Only in a few cases, i.e. *Chlorella fusca* 211-8b, *Chlorella fusca* 211-8p and *Chlorella* sp. 620, which possess a thin trilaminar structure and an exceptionally thick inner cell wall layer (Burczyk, 1982), was the percentage of stained cells a little higher (at most 24%). By contrast, in the eight strains which did not contain algaenan in the cell wall, 100% of the cells were stained.

Discussion

A review of the dyes used for staining algal cells is given by Stadelmann (1962). Numerous dyes, especially those with fluorescent features, have been used to determine the characteristic structures or compounds within algal cells, e.g. DAPI for staining DNA molecules (Mitova et al., 2005), calcofluor white and berberine for the determination of specific types of polysaccharides

(Rodriguez et al., 2000) and Fluostatin-1 as a dye for cell walls (Yamamoto et al., 2003). Tonabene and Benemann (1985) and Cooksey et al. (1987) used Nile Red for the rapid screening of high lipid-containing strains, while Sommerfeld et al. (1987) used this method for screening 3000 algal strains belonging to various taxonomic units, including strains forming algaenan and related biopolymers (Sheehan et al., 1998). It was also reported that there were differences in the brilliant cresyl blue staining of algae belonging to the *Desmidiaceae*, allowing two groups of algae to be distinguished. However, no data concerning the composition of the cell wall in the tested algae are available (Stadelmann, 1962).

The results presented here show that the significant differences in staining between the two types of algal strains investigated were strongly correlated with the presence or absence of algaenan in the cell wall. It might be suggested that the low percentage of stained cells occurring in strains containing algaenan and trilaminar cell wall structure could be caused by the death or damage of the cells, because stained cells are not usually able to grow. However, in contrast to the fluorescent dyes described above, 0.2% crystalline violet, commonly used as a vital dye for staining Gram-positive and Gram-negative bacteria, does not influence the viability of algaenan-containing algal strains. The staining effect remains permanent even after rinsing the cells, which is probably caused by the passive penetration of dye molecules into the algal cells (Stadelmann, 1962).

The trilaminar structure forming the outer cell wall layer of *Chlorococcalean* algae in a number of strains containing algaenan (Burczyk, 1982) seems to be responsible for the differences in staining with crystalline violet described here. Strains which do not contain algaenan are characterized by a homogeneous outer cell wall layer (Burczyk, 1982). The trilaminar structure hinders the penetration not only of the relatively large molecules of crystalline violet (MW 407.5), but also of smaller molecules of detergents, such as linear alkylobenzene sulphate (LAS), dodecylbenzene sulphate (DBS) and Triton X-100 (Biedlingmeier et al., 1987; Corre et al., 1996). The penetration of detergent compounds and dyes probably depends not only on the size of the molecules. The chemical nature of the compounds also plays an important role in this process and in their toxicity to algal cells.

The simple assay described here could be considered as a rapid, inexpensive method to distinguish both non-algaenan and algaenan-containing algal strains. Knowledge on the penetration of dye molecules into the algal cell wall is currently poor, and a large number of 'model dye substances' with known molecular weight and chemical structure will need to be tested in order to identify factors important for the process of vital staining.

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Short communication

VARIETAL CROSS DIALLEL ANALYSIS FOR SEED YIELD AND ITS COMPONENTS IN FENNEL (*Foeniculum vulgare* MILL)

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A half diallel of nine genetically diverse varieties was made and the resulting 36 F_1 populations were evaluated along with the parents. The analysis of variance indicated that heterosis was significant for all the characters, except test weight. The heterosis components were also significant for most of the traits studied. The specific heterosis component accounted for more than 80% of the overall heterosis, indicating a complex type of inheritance for seed yield and its component traits. The crosses JF 29 × Local, RF 125 × JF 29 and UF (M)-1 × RF 101 exhibited significant positive heterobeltiosis for seed yield per plant. The parents RF125 and JF 29 merit attention as parents in the development of hybrids. The use of recurrent selection and the development of composites are suggested as ways to improve yielding ability in fennel.

Key words: fennel, heterosis, heterobeltiosis, varietal diallel, seed yield

Introduction

Fennel (*Foeniculum vulgare* Mill; 2n=22) is an annual, stout, aromatic plant belonging to the Apiaceae family. Two types of fennel are known – bitter fennel and sweet fennel. Indian fennel is of the bitter type. It is an important seed spice crop of high value, having several medicinal properties. Fennel is mainly grown for its seeds, which are used for chewing. All the aerial plant parts are aromatic due to the presence of volatile oil, which is responsible for the pleasant flavour. The volatile oil is also used as a perfume and scenting agent in many preparations (Redgrove, 1933). Fennel is considered to be a native of Southern Europe and the Mediterranean region. It is widely cultivated throughout the temperate and sub-tropical regions of the world, in countries such as Romania, Russia, Germany, France, Italy, India, Japan, Argentina and USA. In India, it is grown on 39,900 ha with a total production of 38,500 tonnes (Menaria and Maliwal, 2007).

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Though fennel has good potential as a cash crop, generating an annual foreign exchange value of Rs. 285 million (2005–06), the crop has remained neglected as far as genetic improvement is concerned. As a result, local types having low productivity and susceptibility to disease are cultivated, resulting in poor production. The yield potential of this crop can be increased by producing hybrid varieties.

Fennel is an allogamous crop with up to 82.2–91.4% cross pollination (Ramanujam et al., 1964), although reports also indicate a high level of self pollination (up to 56% in a single umbel) in isolated plants (Németh et al., 1999). All the existing varieties at S. K. N. College of Agriculture, Jobner were developed using mass selection. Inbreds have not yet been developed in this crop, although the programme for developing inbred lines headed by E. V. Divakara Sastry is in an advanced stage (personal information). Gardner and Eberhart (1966) proposed a varietal diallel for such situations and gave a statistical genetic model which serves as a guide to plant breeders in the design and analysis of experiments aimed at obtaining the maximum amount of useful genetic information concerning a fixed set of random mating varieties (i.e. allogamous population). The present work was undertaken to study the genetics of yield, in order to generate information for use in designing breeding programmes.

Materials and methods

The material consisted of crosses made by intermating nine genetically diverse varieties (maintained by sib mating) namely, RF 125, UF (M) 1, UF 90, RF 101, UF 134, JF 29, HF 71, HF 102 and a local type, in a half diallel fashion. Since fennel is a cross-pollinating species, the crosses were made by dusting pollen collected from randomly selected plants of a male parent on several emasculated umbels of a female parent. The resulting 36 F_1s were grown along with their nine parents in a randomized complete block design with three replications. In each replication, parents and F_1s were sown in a plot 2.0 × 0.9 m in size, accommodating two rows 2 m in length spaced 45 cm apart with an intra-row spacing of 20 cm. All the recommended practices were followed (Sharma et al., 1996).

Observations on height up to main umbel, total plant height, number of branches per plant, umbels per plant, umbellets per umbel, seeds per umbel, biomass per plant, harvest index and seed yield per plant were recorded on ten randomly selected plants from each plot. The data were recorded on a whole plot basis for days to 50% flowering and test weight. The data were subjected to diallel analysis according to model II of Gardner and Eberhart (1966). This model assumes that the parents used are a fixed set of random mating varieties with no epistasis and diverse gene frequencies. The genetic effects are defined as a function of gene frequencies and additive and dominance effects for individual loci.

When parents and their half diallel crosses are grown together, the additive effect (A) and dominance effect (D) are confounding and must therefore be estimated jointly. Under such conditions, depending upon the presence or absence of heterosis and its components, four models are suggested (Gardner and Eberhart, 1966). They are:

1. $Y_{jj'} = \mu_v + \frac{1}{2}(v_j + v_{j'})$
2. $Y_{jj'} = \mu_v + \frac{1}{2}(v_j + v_{j'}) + \delta\bar{h}$
3. $Y_{jj'} = \mu_v + \frac{1}{2}(v_j + v_{j'}) + \delta\bar{h} + \delta(h_j + h_{j'})$
4. $Y_{jj'} = \mu_v + \frac{1}{2}(v_j + v_{j'}) + \delta(\bar{h}) + \delta(h_j + h_{j'}) + S_{jj'}$

where

$Y_{jj'}$ = Mean of a cross between j and j'

μ_v = Mean of all of parental varieties included

v = The variety effect when parent varieties are included in the analysis, as done in the present case

$\bar{h}$ = Average heterosis; h_j and $h_{j'}$ refer to varietal heterosis and $S_{jj'}$ refers to the specific combining ability of the cross between j and j'

$\delta = 0$ when $j = j'$ and 1 when $j \neq j'$

Results and discussion

The analysis of variance indicated significant differences for most of the traits, revealing the existence of variability between the parents and their hybrids. The parents vs F_1s interaction was also significant, indicating the existence of heterosis. This is also supported by the results obtained from the partitioning of variation due to entries into varieties and heterosis (Table 1). This partitioning further indicated the importance of heterosis in the inheritance of the traits. This component accounted for more than 80% of the total variation. This was true even for the character test weight, for which heterosis was non-significant, but the contribution was more than 85% of total variation. This indicates that all the characters were controlled by additive, dominance and epistatic components (Bailey et al., 1980). Further, the partitioning of overall heterosis indicated that the contribution of specific combining ability was considerably higher (Table 2). This suggested that the data would fit to model 4. This supposition was found to be correct, supporting the observation of complex inheritance, including additive, dominance and epistatic components. These genetic components cannot be estimated separately in model II (Gardner and Eberhart, 1966) because of the confounding effect.

On the basis of *per se* performance, UF 134, HF 102 and RF 125 were superior (Table 3). A comparison of the mean values of seed yield with the mean values of other morphological traits indicated that the parents and hybrids showed similarity in pattern, i.e. parents and hybrids which were superior for seed yield were also superior for seeds per umbel followed by branches per plant, total plant height and umbellets per umbel. Hence improvement in seed yield could be expected even when selection was based on the components of seed yield.

The sign of the average effect, $\bar{h}$, is generally dependent upon the distribution of genes in the parents and the difference between the heterozygote and the mid-parent value at any given locus. It was negative and non-significant (–0.52) for seed yield per plant, which is undesirable. Based on the $S_{jj'}$ effects, which represent the SCA effects in Griffing's (1956) notation, the crosses JF 29 × Local, RF 125 × JF 29 and UF (M)-1 × RF 101 were best, having desirable and significant $S_{jj'}$ effects for seed yield per plant. These crosses also showed superior heterobeltiosis.

Table 1
Analysis of variance and the extent of heterosis observed for different characters in fennel

Character	Source of variance		Error	Heterosis as % of total variance
	Variety (v_j)	Heterosis ($h_{jj'}$)		
Days to 50% flowering	8.59	20.71**	7.41	91.56
Height up to main umbel	418.94*	395.45**	157.46	80.94
Total plant height	257.49	379.94**	128.49	86.91
Branches per plant	2.94	2.43*	1.51	78.82
Umbels per plant	152.43**	137.16**	15.55	80.19
Umbellets per umbel	16.05	21.10**	10.93	85.54
Seeds per umbel	12680.91*	13827.56**	5036.61	83.07
Test weight	0.17	0.31	0.49	89.05#
Biomass per plant	407.96*	529.33**	148.59	85.38
Harvest index	53.93**	31.58**	17.08	72.49
Seed yield per plant	31.05**	45.13**	7.37	86.74

Mean squares not significant at P =0.05; *, **Significant at P =0.05 and P =0.01, respectively

Table 2
Analysis of variance for heterosis components for different characters in fennel

Character	Average ($\bar{h}$)	Variety (h_j)	SCA ($S_{jj'}$)
Days to 50% flowering	287.53** (38.57)	12.08 (12.97#)	13.38* (48.47)
Height up to main umbel	590.00 (4.14#)	515.82** (28.99)	352.58** (66.87)
Total plant height	203.12 (1.49#)	436.74** (25.54)	369.66** (72.97)
Branches per plant	0.00 (0.00#)	4.77** (43.55)	1.83 (56.45#)
Umbels per plant	13.79 (0.28#)	129.61** (20.99)	143.96** (78.72)
Umbellets per umbel	25.62 (3.37#)	25.28** (26.62)	19.69* (70.00)
Seeds per umbel	15919.23 (3.19#)	19975.05** (32.10)	11928.61** (64.70)
Test weight	0.37 (3.27#)	0.29 (21.09)	0.31 (75.63#)
Biomass per plant	15.93 (0.08#)	765.21** (32.12)	478.46** (67.79)
Harvest index	4.23 (0.37#)	40.77* (28.69)	29.87* (70.94)
Seed yield per plant	5.79 (0.36#)	32.45** (15.98)	50.34** (83.66)

Figures in parenthesis represent percentage of $h_{jj'}$ mean squares; #: Mean squares not significant at P =0.05; *, **: Significant at P =0.05 and P =0.01, respectively

Table 3
Mean seed yield (g plant^{-1}) and rank of the parents, the mean rank over different characters, the varietal effect and the heterotic effect in fennel

Parent	Seed yield	Rank	Mean rank	Varietal effect (v_j)	Heterotic effect (h_j)
RF 125	17.64	3	4.5	1.45	1.45
UF (M) 1	15.76	5	4.6	–0.43	0.93
UF 90	12.67	8	4.9	–3.52	0.98
RF 101	15.09	6	5.5	–1.10	–0.49
UF 134	21.90	1	3.5	5.71	–1.84
JF 29	12.10	9	5.9	–4.09	2.49
HF 71	17.61	4	5.7	1.42	–2.90
HF 102	19.25	2	4.2	3.06	– 1.53
Local	13.70	7	5.3	–2.49	0.93
Mean				16.19 (μv)	–0.52
S.E.				2.56	±1.53

The parents RF 125 and JF 29 were superior based on v_j and h_j values. These two parents appeared in the majority of crosses exhibiting higher S_{jj}, and heterobeltiosis estimates. The other parents worth considering were UF (M) 1 and RF 101.

The parents and crosses which showed superiority for seed yield were also superior for biomass per plant and seeds per umbel, followed by branches per plant, total plant height and umbellets per umbel. Hence, improvement in yield can be expected even when selections are based on these component traits.

A high amount of heterosis exists for various traits, including seed yield, as revealed by the present study, so the development of hybrid varieties holds promise. RF125 and JF 29 merit attention as parents in the development of hybrids. Crossing in fennel is difficult due to the small size of the flower. Hence, the use of recurrent selection and the development of composites are suggested to improve the yielding ability in fennel. However, with the availability of inbreds and with use of the rapid emasculation technique proposed by Singh et al. (2000), the development of hybrids should become easier.

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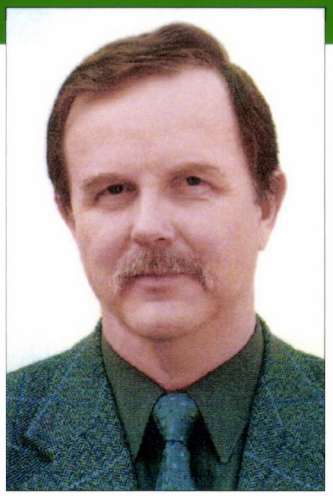

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EFFECT OF SOWING DATE ON THE YIELD AND QUALITY OF MAIZE HYBRIDS WITH DIFFERENT GROWING SEASONS

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The yield, protein and starch content of Martonvásár maize hybrids belonging to different FAO groups were examined in experiments involving early, optimal and late sowing dates in two different years (drought – 2007, favourable water supplies – 2008) on a calcareous chernozem soil with loam texture at the Látókép Experimental Station of the Centre of Agricultural Sciences and Engineering, University of Debrecen.

Sowing date had a significant effect on maize grain yield in the dry year. The grain yields of hybrids with longer growing periods were significantly higher than those with shorter growing periods in both years, but they reacted sensitively to the change in sowing date in the dry year. Due to the rainfall distribution in the growing season, sowing date did not modify the performance of the hybrids in the year with favourable water supplies. Sowing date had a significant effect on the grain protein content in the dry year, with significantly higher values after late sowing than after early or optimal sowing. Averaged over the sowing dates, the protein content of the FAO 200 hybrid was significantly higher in both years than that of hybrids in other FAO groups. In the dry year, the greatest difference in protein content could be observed between the early and late sowing dates for hybrids in all four FAO groups. A negative correlation was found between yield and protein content. Sowing date significantly increased the starch content of maize in the favourable year, with a significant difference between early and late sowing dates.

In the dry year higher starch contents were recorded for all the hybrids and for all the sowing dates than in the favourable year. In the dry year, sowing date only caused a significant difference in the starch content in the case of FAO 200 sown at optimal and late sowing dates. In the favourable year, a significant difference was only obtained for the starch content of the FAO 400 hybrid sown at early and late sowing dates. Satisfactory quality can only be achieved if suitable genotypes are grown with appropriate technologies.

Key words: maize, sowing date, year

Introduction

When selecting the correct sowing date for maize, various factors should be considered, including the temperature during the growing season, soil texture, geographical location, weed cover, soil infection by pests and pathogens, seed quality, heat requirements during sprouting and development, the hybrid maturity group, the aim of production and the sowing technology.

The results of a sowing date experiment carried out in Martonvásár, Hungary showed that the yield of maize sown in mid-April was 7% higher than after sowing in mid-May, averaged over several years. Sowing one month later also delayed ripening by 11–16 days (14 on average) (Berzsenyi et al., 1998).

The average temperature on the sowing date was reported to affect the duration of sprouting (Győrffy et al., 1965). Marton (1991) considered a temperature range of 9–18°C to be adequate for evaluating and comparing the cold resistance. Cold conditions during sprouting and seedling development result in the plants becoming yellow, retarded development, and later flowering, yield formation and cob ripening.

The starch content of maize is around 70–75%, making it an excellent energy resource. Its quality and forage value are basically determined by the protein and oil content of the grain, and by the amino acid and fatty acid composition. Although quality parameters are hereditary, they may be modified by ecological and agrotechnical factors (Pásztor and Kováts, 1985; Nagy, 1997).

The distribution of proteins and protein fractions is uneven in the grain, so factors that determine grain weight and the ratio of grain components also affect the grain protein content. As with other cereals, there is a negative correlation between the quantity and protein content of the grain yield (Bhatia and Rabson, 1987; Sander et al., 1987). Many factors influence the quality of crop products which serve as a basis for food and forage, the most important of which are the variety/hybrid (as biological factors), climatic factors, and the production technology.

Szirtes et al. (1977) stated that the crude protein content of the grain yield was significantly determined by the weather. This change was in close correlation with yield fluctuation. Kralovánszky (1975) observed a slight negative correlation between the average yield of maize and its crude protein content, while they found a strong positive linear correlation between average yield and crude protein yield.

According to Szániel et al. (1980) and Lilburn et al. (1991), protein content is lower in wet years and higher in dry years. The most influential factors are heat units and the quantity and distribution of precipitation in June, July and August (Asghari and Hanson, 1984). Győri and Sipos (2005) examined the protein content of different hybrid genotypes between 2002 and 2004, and found that the decrease in protein content at higher water supplies (from rainfall or irrigation) could be corrected by proper nutritional management, as no dilution was observed for the given hybrids.

Materials and methods

The study was carried out on calcareous chernozem soil with loam texture at the Látókép experimental station of the University of Debrecen in 2007 and 2008.

Experimental site

Soil analysis data from 2002 showed an average soil pH of 6.6, which is optimal for crop nutrient uptake. The soil texture was medium-heavy loam. The upper (0.2 m) soil layer had a soil plasticity of 3.7 K_A and a total salt content of 0.05% m/m. The $CaCl_2$ content in the upper 0.8 m of soil was 0% m/m (i.e. there was lime deficiency), but it steeply increased to 11% m/m between 1 m and 1.6 m (i.e. moderately limy). The lime layer was thus at a much lower depth compared with the 1984 data. The humus layer has decreased due to intensive cultivation during the last 23 years; it is currently 2.4% m/m in the upper 0.2 m of soil, whereas it does not exceed 1% m/m at a depth of 1.2 m. The soil nitrogen and potassium supply was good and the phosphorus supply was average.

Weather

Environmental parameters were continuously monitored by an automatic measurement and data-logging station. Air temperature (°C) at heights of 0.5, 1 and 2 m, relative humidity (%), soil temperature (°C) at depths of 50, 250 and 500 mm, incoming radiation (W/m^2) and the amount of precipitation (mm) were measured every sixth second. The statistical parameters derived from the data (average, standard deviation) were stored every 15 minutes. Basic data were accompanied by pheno- and phytometric observations and soil analyses.

The extreme weather conditions in 2007, with temperatures of 40°C for several days in July, accompanied by a complete lack of rainfall, had a devastating effect on the maize. Mean monthly temperatures were higher than the long-term average for a whole year (Sept. 2006 to Aug. 2007), leading to an effective heat unit sum of 1624°C. The potential evapotranspiration in 2007 was 899 mm, of which 651 mm would have been needed for the evaporation from crops. This amount exceeded the rainfall sum (Apr. to Sept.) by 370 mm. The difference between the precipitation and PET value was 453 mm. Altogether, this dry year was unfavourable for maize growing (Szász, 1973).

The total rainfall sum (484 mm) in the 2008 growing season was sufficient for maize. The distribution was also favourable, with 286 mm in June and July. The year was thus wetter than average. The total heat sum in the growing period was 1677°C. The yearly potential evapotranspiration was 721 mm, only 22 mm more than the yearly precipitation (699 mm). The rainfall sum in the growing season was greatly exceeded by the PET value (580 mm) for the same period, making 2008 an optimal year for maize production.

The two-factorial experiment was set up in a split plot design with four replications. Sowing took place on three dates: ten days earlier than the optimal date (early), on the optimal date (24th April) and fifteen days after the optimal date (late). After the forecrop harvest the area was ploughed to a depth of 27 cm.

The yield, protein and starch content data were recorded for Martonvásár hybrids belonging to different FAO groups (Mv 251, FAO 200; Mv Tarján, FAO 300; Mv Koppány, FAO 400; and Mv 500, FAO 500).

The grain starch and protein content were measured using a Foss Intratec instrument, which operates on the near infrared spectroscopy (NIR/NIT) principle and allows measurements to be made on the whole grain in less than a minute.

The data were evaluated using the general linear model (GLM) of SPSS for Windows 13.0 to express the effect of treatments on yield, protein and starch content. Mean values were compared using the least significant difference method ($LSD_{5\%}$) and homogeneous groups were formed using Duncan's multiple range test, correcting confidence intervals using Bonferroni's method to avoid alpha error. Yields within the homogeneous groups did not differ from each other at the 5% significance level.

Results and discussion

The effects of sowing date and year on the yield, starch and protein content of Martonvásár maize hybrids, and correlations between these factors, were examined in two significantly different years (2007 and 2008). The weather in 2007 was unfavourable for maize yield formation, whereas 2008 was optimal for both vegetative and generative development.

Effect of sowing date on the yield of Martonvásár maize hybrids

Variance analysis showed that both sowing date and hybrid type significantly influenced the maize yield, but the effect of the year was the most significant on the basis of mean square deviation (MS) values. There was no significant interaction between sowing date and hybrids. Nevertheless, the year modified the effects of both hybrid and sowing date (Table 1).

The variance analysis for a two-factorial split plot design was performed on the grain yield each year (Table 2). Based on the values of MS, sowing date had the most significant effect in the dry year of 2007, whereas it was not significant in the year with favourable weather (2008). The effect of hybrid was statistically significant at a level of 0.1% in both years. The interaction between hybrids and sowing date was significant in 2007, i.e. the effect of the hybrid changed with the sowing date, but the interaction was not significant in 2008.

Analysis of the sowing date experiment using the LSD test showed that the highest yield in 2007 was obtained at the optimal sowing date (6.715 t ha^{-1}), a surplus of 1400 kg ha^{-1} ($P<0.001$) compared with late sowing and 369 kg ha^{-1} compared with early sowing, though the latter was not statistically significant (Fig. 1). The yield of early sown maize was significantly higher (6.346 t ha^{-1}, $P<0.01$) than that of late sown maize (5.315 t ha^{-1}). In 2008 the highest yield (9.074 t ha^{-1}) was recorded for early sown maize, but this was not significantly different from the other sowing dates at the 5% level on the basis of Duncan's test.

Table 1

Variance analysis on the yields (t ha^{-1}) in the sowing date experiment in different years (Debrecen, 2007–2008)

Factors	Mean squares	Degrees of freedom	F value
Hybrid (A)	13.077	3	43.7***
Error (a)	0.299	9	
Sowing date (B)	5.705	2	10.8*
Error (b)	0.529	6	
Year (C)	184.415	1	49.8**
Error (c)	3.706	3	
A × B	0.311	6	1.2^{ns}
Error (a × b)	0.252	57	
A × C	0.796	3	3.2*
Error (a × c)	0.252	57	
B × C	3.174	2	12.6***
Error (b × c)	0.252	57	

***, **, *: Significant at the P=0.1%, 1%, and 5% levels, respectively; ^{ns} = non-significant

Table 2
Variance analysis results on the effect of sowing date and hybrid on the yield (t ha^{-1})
(Debrecen, 2007– 2008)

Factors	2007			2008		
	MS	DF	F value	MS	DF	F value
Hybrid (A)	4.569	3	54.0***	9.304	3	25.4***
Error (a)	0.085	9		0.366	9	
Sowing date (B)	8.425	2	8.5*	0.454	2	1.3^{ns}
Error (b)	0.994	6		0.347	6	
A × B	0.408	6	2.8*	0.223	6	1.1^{ns}
Error (a × b)	0.145	18		0.200	18	

***, * Significant at the P = 0.1% and 5% levels, respectively; ^{ns} = non-significant

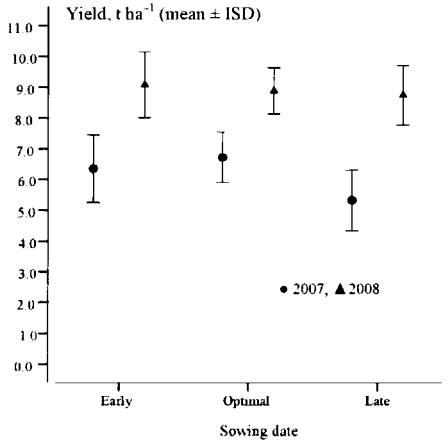

Fig. 1. Effect of sowing date on maize yield (Debrecen, 2007–2008)

In the dry year the FAO 400 hybrid had the highest grain yield (6.584 t ha^{-1}), but this was not significantly different from that of FAO 300 and FAO 500. The FAO 200 hybrid yielded significantly less than the FAO 400 ($P<0.01$) and FAO 500 ($P<0.01$) hybrids, but the yield was not significantly different from that of FAO 300. Significant differences were observed between the hybrids in the favourable year (Fig. 2). FAO 200 had the lowest yield (7.822 t ha^{-1}), which was significantly different at the 0.1% level from the other hybrids. FAO 400 yielded 676 kg ha^{-1} more than FAO 300 ($P<0.01$). FAO 500 had the highest yield (9.864 t ha^{-1}), exceeding that of FAO 300 by 1250 kg ha^{-1} ($P<0.001$) and that of FAO 400 by 575 kg ha^{-1} ($P<0.05$). It could thus be seen that hybrids with longer maturity periods (FAO 400, FAO 500) tend to have significantly higher grain yields ($P<0.001$) than those with shorter growing periods (FAO 200, FAO 300).

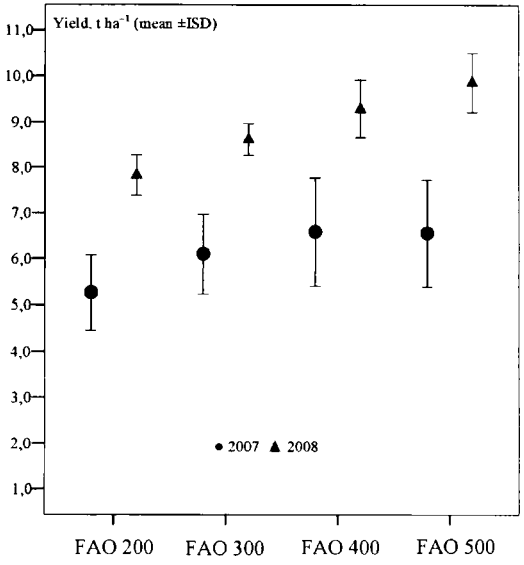

Fig. 2. Effect of year on the grain yield of maize hybrids (Debrecen, 2007–2008)

The effect of sowing date on the yield of maize hybrids was different in each year (Fig. 3). In the dry year the highest yield was obtained with the optimal sowing date (24th April) for all four hybrids. For the FAO 200 and FAO 300 hybrids these differences were not significant, but for the FAO 400 and FAO 500 hybrids late sowing caused a significant yield drop (1916 kg ha^{-1}, $P<0.05$; 1814 kg ha^{-1}, $P<0.05$, respectively) in comparison with the optimal sowing date. Hybrids with longer vegetation periods (FAO 400, FAO 500) thus reacted more sensitively to changes in sowing date in the dry year. Due to the satisfactory rainfall distribution during the growing season in 2008, sowing date had no significant effect on the yields of the maize hybrids.

Effect of sowing date on the protein and starch content of maize grain

The protein content of the grain ranged from 9.4%–12.6%, depending on the sowing date and crop year, with the highest value in the case of late sowing in the unfavourable year of 2007. This was significantly different from the values measured for the early ($P<0.01$) and optimal ($P<0.05$) sowing dates, but there was no significant difference between the latter. In 2008 there was no significant difference in protein content between the three sowing dates on the basis of Duncan's test (Fig. 4).

The protein content of FAO 200 was significantly higher ($P<0.001$) than that of the other hybrids averaged over the sowing dates. There was no significant difference between the protein content values of FAO 300, FAO 400 and FAO 500 in 2007, whereas there was a statistically significant difference ($P<0.01$) between FAO 500 (9.3%) and FAO 300 (8.7%) in 2008.

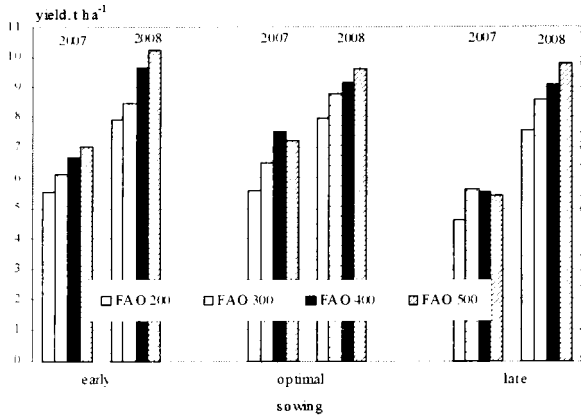

Fig. 3. Effect of sowing date and maturity group on the grain yield of maize (Debrecen, 2007– 2008)

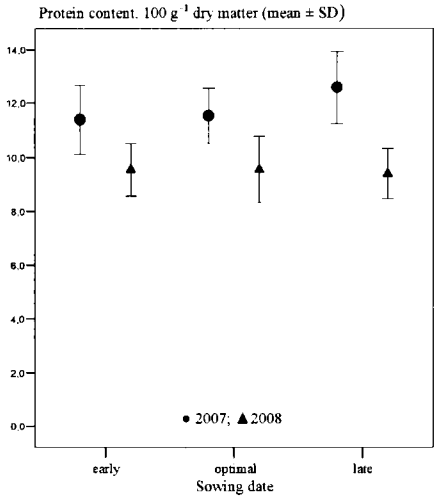

Fig. 4. Effect of sowing date on the protein content of maize grain (Debrecen, 2007–2008)

In the dry year lower protein content was recorded in early sown hybrids than in those sown at the optimal sowing date, except in the case of FAO 200, but this difference was not significant. The biggest difference in protein content was measured between the early and late sowing dates, with significantly higher values for late-sown hybrids: FAO 200 ($P<0.1$), FAO 300 and FAO 400 ($P<0.01$) and FAO 500 ($P<0.5$). The protein contents were only higher after late sowing than at the optimal sowing date for FAO 200, FAO 400 ($P<0.001$) and FAO 300 ($P<0.01$). In 2008 none of the hybrids exhibited a significant change in protein content as a result of the sowing date (Fig. 5).

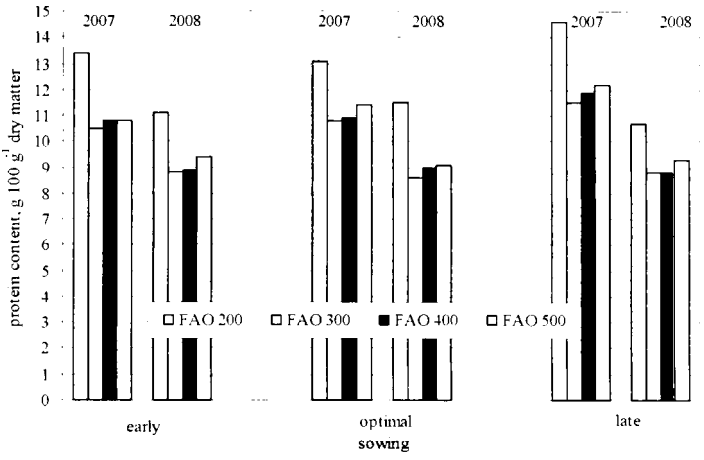

Fig. 5. Effect of sowing date and maturity group on the protein content of maize (Debrecen, 2007–2008)

Variance analysis was performed to examine the effect of sowing date, hybrids and years on the protein content of maize. The MS values indicated that the year had the most significant effect, while a consistent difference was also observed for the sowing date. There were significant differences between the hybrids (Table 3).

Kralovánszky (1975) observed a negative correlation between yield and protein content. The present experiments also showed that protein content was lower in the year when a high yield was obtained (2008) (Fig. 6).

Table 3
Variance analysis on the effect of sowing date and year on the protein content (g 100 g^{-1} dry matter; Debrecen, 2007–2008)

Factors	Mean square	Degrees of freedom	F value
Hybrid (A)	32.864	3	149.2***
Error (a)	0.220	9	
Sowing date (B)	2.470	2	9.2*
Error (b)	0.268	6	
Year (C)	131.517	1	490.7***
Error (c)	0.268	3	
A × B	0.037	6	0.2^{ns}
Error (a × b)	0.230	57	
A × C	0.262	3	1.1^{ns}
Error (a × c)	0.230	57	
B × C	4.348	2	18.9***
Error (b × c)	0.230	57	

***, * Significant at the P=0.1% and 5% levels, respectively; ^{ns} = non-significant

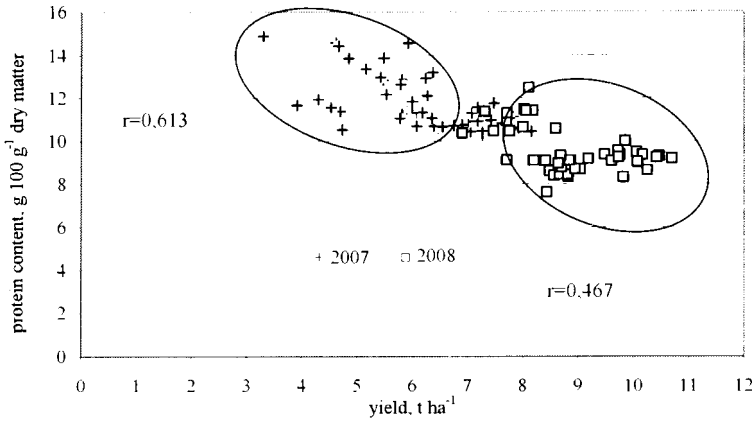

Fig. 6. Correlation between the yield and protein content of maize (Debrecen, 2007–2008)

Higher starch content was recorded in 2007 than in 2008. Duncan's test at the 5% level showed no significant difference between the three sowing dates in the dry year, whereas in the favourable year there was a significant difference ($P<0.01$) between the early (70.9%) and late sowing dates (72.9%). The starch content values of the hybrids did not differ significantly in 2007, whereas in 2008 the starch content of FAO 500 was significantly higher ($P<0.05$) than that of FAO 200 (Table 4).

Table 4
Effect of sowing date on the starch content of maize grain
(Debrecen, 2007–2008)

Sowing dates and FAO groups	Starch content (g 100 g^{-1} dry matter)	
	2007	2008
Effect of sowing date (averaged over hybrids)		
Early	73.7a	70.9a
Optimal	73.9a	71.8a,b
Late	74.5a	72.9b
	74.0	71.9
Effect of hybrids (averaged over sowing dates)		
FAO 200	73.3a	70.9a
FAO 300	74.2a	71.6a,b
FAO 400	74.3a	71.9a,b
FAO 500	74.3a	73.1b
	74.0	71.9

Data marked with the same letter within a column are not significantly different based on Duncan's multiple range test

Variance analysis showed that sowing date did not influence the starch content of maize grain in the dry year, whereas it had a significant ($P<0.05$) effect in the favourable year. The hybrid had no significant effect on starch content in either year. The sowing date × hybrid interaction was significant ($P<0.05$) in 2007.

In the dry year higher starch content was observed for all sowing dates and all hybrids than in 2008 (Fig. 7). In 2007, the starch content of the hybrids was not significantly influenced by sowing date, except for FAO 200, where there was a significant difference ($P<0.05$) between the optimal and late sowing dates. The highest starch contents were measured for all the hybrids at the late sowing date in 2008. Sowing date had no significant effect on the starch content of the hybrids except for FAO 400, where a significant difference ($P<0.05$) was observed between the early and late sowing dates.

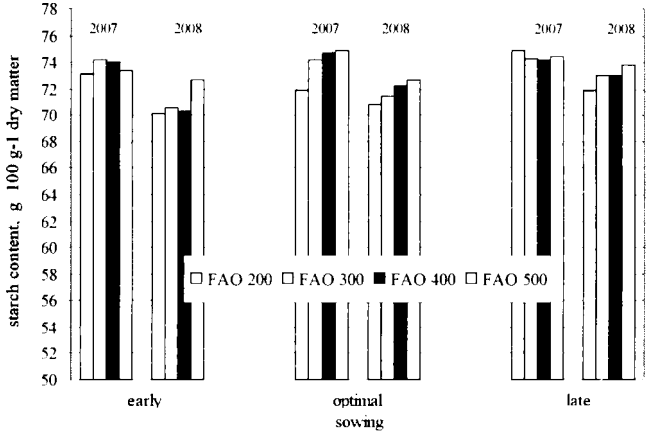

Fig. 7. Effect of sowing date and maturity group on the starch content of maize grain (Debrecen, 2007–2008)

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USE OF GENETIC MARKERS IN THE INVESTIGATION OF STARCH CONTENT IN MAIZE

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The chemical composition of maize makes it suitable for a wide range of non-conventional uses, including utilisation as a new source of energy for the 21st century as a raw material for biofuel. The aim of the experiments was to amalgamate the application of genetic markers with conventional breeding methods to produce maize hybrids whose starch content and ecostability satisfied the demands of industrial use, while having yield potential and agronomic traits on a par with those of hybrids currently cultivated. The chemical quality of 220 lines was evaluated using the NIR spectrometric technique, and the five maize inbred lines with the lowest and highest starch contents were selected for genetic marker studies. The variety identification of the lines was carried out using the isoenzymes stipulated by UPOV. The following SSR (simple sequences repeat) markers were tested: phi 095, umc 1057, nc 004, phi 096, nc 007, umc 1564, phi 85, y1 SSR, umc 1178, nc 009, phi 070, umc 1066, umc 1741, umc 1069, phi 033, phi 061, wx, phi 032, phi 084 and phi 062. The analysis of the fragment patterns revealed three SSR markers that appeared to be correlated with the starch content of the maize lines. These were the primer pairs *y1 SSR, umc 1069* and *phi 062*. These results are only of a preliminary nature, however, as the incorporation of starch is probably regulated by several genes, and the studies suggest it is also influenced by several environmental factors. It also appears likely that the bioethanol yield is determined not only by the starch content, but also by other parameters. Further research should thus be expanded to include investigations into the structural and fermentability traits of starch molecules, including the characterisation of these traits using genetic markers.

Key words: maize, starch content, bioethanol, NIR spectroscopy, SSR markers

Introduction

In Hungary the most promising raw material for biofluel is maize, the chemical composition of which allows it to be used for a wide range of non-conventional purposes (Torney et al., 2007). Among the fodder crops cultivated

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in Hungary, maize has the greatest starch content and the lowest crude protein content. The kernel starch content is accumulated in the endosperm, which also contains 80% of the total protein content of the kernels. If water supplies after flowering are optimum (rainfall, irrigation), more starch is accumulated in the kernels than in dry years. As a consequence of the greater carbohydrate accumulation, there is an increase in the total yield, but a reduction in the protein content of the kernels.

As a new source of energy and industrial raw material for the 21st century, maize allows bioethanol to be produced from starch of plant origin at optimum cost and with the desired composition, while in the following phase the by-products of the technology can also be utilised as fuel for vehicles or heating. For this purpose the quality traits of existing hybrids must be evaluated and improved to suit the starch requirements of bioethanol production. The aim of the present experiments was to use a combination of genetic markers and conventional breeding methods to develop maize hybrids whose starch content and ecostability were suitable for industrial use, while having yield potential and agronomic traits similar to those of the hybrids currently cultivated (James, 2006). A further important breeding aim was to improve resistance to abiotic stress and to the pathogens and pests prevalent in Hungary, thus increasing yield stability.

Data in the literature indicate a close correlation between thousand-kernel mass and quality parameters. Both these data and the results of our own work suggest that early-maturing maize lines of the flint type have higher protein and oil contents than starch contents. The kernels are small, with low thousand-kernel mass and below-average starch content. Lines with high starch content, suitable for the development of high-starch hybrids, tend to be of the dent type, which mature later and have large kernels (Hegyi et al., 2008).

Data from the literature confirm that SSR markers can be effectively used to characterise various traits such as starch content using genetic markers (Sherry et al., 2002; Gethi et al., 2002).

The research is expected to result in new hybrids with higher starch contents and better ecological stability, irrespective of the technology and environment. When these new hybrids are introduced into cultivation there should be an improvement in quantitative and qualitative yield parameters and it should be possible to produce larger, more homogeneous lots of maize that can be more easily handled by the energy industry.

Materials and methods

Plant material

A total of 220 maize inbred lines were sown one to a row in a small-plot experiment arranged in a random block design with four replications at two locations.

Quality analysis

The analysis was carried out using a Fourier transform near infrared (NIR) spectrometer (Bruker Optics GmbH, Ettlingen, Germany).

Variety identification of the lines

Based on the quality analysis results, the inbred lines with the 5 lowest and 5 highest starch contents were chosen for genetic marker analysis. The variety identification of the lines was carried out by means of starch gel electrophoresis using the isoenzymes stipulated by UPOV: MDH (malic acid dehydrogenase, 6 loci), IDH (isocitrate dehydrogenase, 2 loci), PGD (6-phosphogluconate dehydrogenase, 2 loci), PGM (phosphoglucomutase, 2 loci), PGI (phosphoglucose isomerase, 1 locus), ACP (acid phosphatase, 1 locus) and ADH (alcohol dehydrogenase, 1 locus), as described by Goodman and Stuber (1983) and Stuber et al. (1988).

Determination of alleles

The genetic interpretation of the zymograms was carried out as suggested by Cardy et al. (1980). By evaluating enzyme polymorphism it was possible to exclude plants originating from cross-pollination.

DNA isolation

A DNA pool was created from the DNA isolates of five seedlings for each variety for genetic marker analysis (Dweikat et al., 1994; Gyulai et al., 2000).

SSR marker analysis

Gene-linked SSR primer pairs 16–18 bp in length were used for the analysis of genetic polymorphism in repetitive DNA sequences (Weining and Langridge, 1991). The primer pairs can be found in the "Maize Cooperation in Genomics and Genetics" MaizeDB database at www.agron.missouri.edu (Table 1).

Determination of polymorphism

The evaluation was carried out using a presence-absence data matrix for the alleles of PCR fragments exhibiting polymorphism (Gyulai et al., 2000).

The values of the polymorphic index content (PIC), which expresses the degree of polymorphism, were determined using the formula $1-\Sigma f_i^2$, where f_i represents the frequency of the i^{th} allele at the given locus (Smith et al., 1997).

Results and discussion

Evaluation of quality data

The NIR spectrometric data showed that the starch content of the lines ranged from 60.31–76.89% (Fig. 1). A large proportion of the lines had values close to the average (70%), but lines were also found with very low (60–65%) and very high (75–76%) starch contents, suitable for the genetic analysis. Although the h^2 value for starch content was lower than that of other quality parameters (e.g. oil and protein content), genotypes with high starch content can be selected by means of regular quality analysis, allowing the trait to be genetically fixed so that it is inherited in hybrids produced from parental lines with high starch content. If these hybrids are grown under satisfactory conditions (irrigation, sufficient rainfall), a favourable starch content can be ensured.

Table 1
Microsatellite (SSR) markers, their repetitive sequences, and the chromosomal localisation of the genes they surround

SSR marker	Linked gene locus	Chromosome localisation	Repetitive sequence
phi 095	*p1* (pericarp colour)	1.03	(AG)x
umc 1057	*cko1* (cytokinin-oxidase-1)	3.02	(CGG)6
nc 004	*adh2* (alcohol-dehydrogenase-2)	4.03	(AG)x
phi 096	*zp1* (zein alpha protein-1)	4.04	(AGGTG)x
nc 007	*ohp2* (opaque-2 heterodimerising protein)	5.01	(CCT)x
umc 1564	*rps15* (S 15 ribosomal protein)	5.03	(CAG)5
phi 85	*gln4* (glutamine-synthetase-4)	5.06	(AACGC)x
y1SSR	*y1* (yellow endosperm)	6.01	CAA; CCATC; CATC
umc 1178	*mir2* (maize insect resistance-2)	6.02	(GGC)6
nc 009	*pl1* (purple plant colour-1)	6.04	(AG)x
phi 070	*mlg3* (LEA seed protein group-3)	6.07	AGCTG
umc 1066	*op2* (opaque endospermium protein-2)	7.01	(GCCAGA)5
umc1741	*rps28* (S 28 ribosomal protein)	8.03	(TC)7
umc 1069	*gst1* (glutathione-S-transferase-1)	8.08	(GGAGA)
phi 033	*sh1* (shrunken-1)	9.01	(AAG)x
phi 061	*wx1* (waxy-1)	9.03	TTCT-GTAT
*	*wx**	*	*
phi 032	*sus1* (sucrose-synthase-1)	9.04	(AAAG)x
phi 084	*nac1* (NaCl stress protein-1)	10.04	(GAA)x
phi 062	*mgs1* (pollen specificity-1)	10.04	(ACG)x
phi 095	*p1* (pericarp colour)	1.03	(AG)x
umc 1057	*cko1* (cytokinin-oxidase-1)	3.02	(CGG)6
nc 004	*adh2* (alcohol-dehydrogenase-2)	4.03	(AG)x
phi 096	*zp1* (zein alpha protein-1)	4.04	(AGGTG)x
nc 007	*ohp2* (opaque-2 heterodimerising protein)	5.01	(CCT)x
umc 1564	*rps15* (S 15 ribosomal protein)	5.03	(CAG)5
phi 85	*gln4* (glutamine-synthase-4)	5.06	(AACGC)x
y1SSR	*y1* (yellow endosperm)	6.01	CAA; CCATC; CATC
umc 1178	*mir2* (maize insect resistance-2)	6.02	(GGC)6
nc 009	*pl1* (purple plant colour-1)	6.04	(AG)x
phi 070	*mlg3* (LEA seed protein group-3)	6.07	AGCTG
umc 1066	*op2* (opaque endosperm protein-2)	7.01	(GCCAGA)5
umc 1741	*rps28* (S 28 ribosomal protein)	8.03	(TC)7
umc 1069	*gst1* (glutathione-s-transferase-1)	8.08	(GGAGA)
phi 033	*sh1* (shrunken-1)	9.01	(AAG)x
phi 061	*wx1* (waxy-1)	9.03	TTCT-GTAT
*	*wx**	*	*
phi 032	*sus1* (sucrose-synthase-1)	9.04	(AAAG)x
phi 084	*nac1* (NaCl stress protein-1)	10.04	(GAA)x
phi 062	*mgs1* (pollen specificity-1)	10.04	(ACG)x

*Chromosomal localisation and sequence of the marker are unknown

The maize inbred lines with the 5 highest and 5 lowest starch contents were chosen on the basis of the NIR spectrometric analysis for testing with SSR markers.

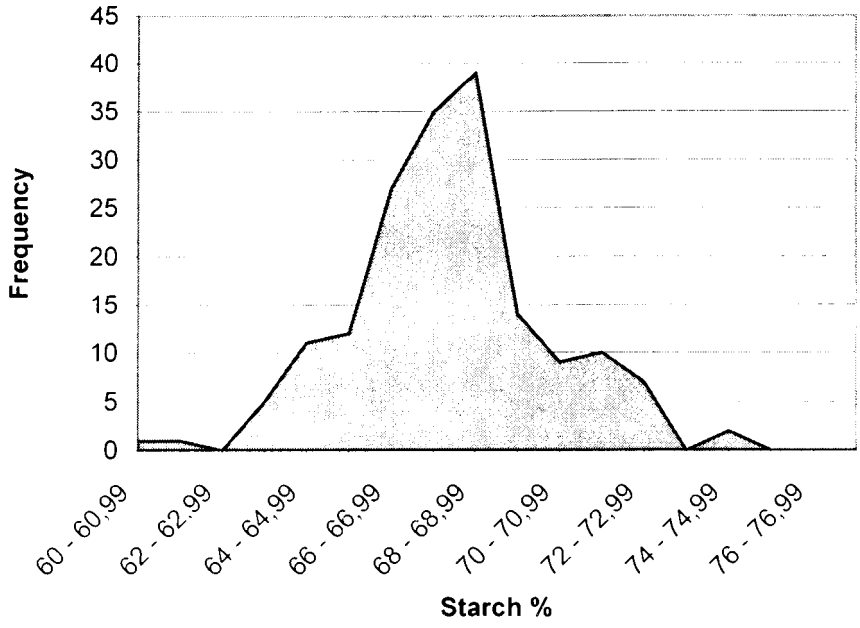

Fig. 1. Distribution of lines as a function of starch content

Analysis of the lines using SSR markers

Polymorphism analysis on the basis of polymorphism index content (PIC)

The applicability of genetic markers to analyse a trait depends on the extent of polymorphism detectable with the given marker. It is thus necessary to establish the polymorphic index content (PIC), which expresses the ability of a locus to discriminate between the lines.

The PIC values of polymorphic SSR markers were high, ranging from 0.04–0.90 (mean value 0.67) (Table 2), indicating that SSR markers could be efficiently applied to detect polymorphism even with a relatively low number of primers.

Among the SSR markers, the highest PIC values ranged from 0.04 for umc 1178 to 0.90 for phi 084. The high values for SSRs confirm data in the literature, indicating that SSR markers are extremely efficient tools for polymorphism analysis (Brown et al., 1996; Smith et al., 1997).

Among the 20 markers tested, the primer pairs wx, phi 061, umc1057 and nc 004 did not give evaluable PCR patterns. The DNA sections amplified by markers phi 096 and phi 095 exhibited monomorphic patterns. The approx. 98% monomorphic pattern of SSR marker umc 1178 was probably due to the recognition of semi-conservative gene regions. Although the primer pairs nc 007 and phi 070 exhibited polymorphism, the differences between the fragments

were so small (a few bp) that the patterns could not be determined unambiguously, so they were not included in the evaluation. The primer pairs phi 084, phi 062, nc 009, phi 85 and umc 1066 exhibited extremely selective polymorphism between the lines (Fig. 2).

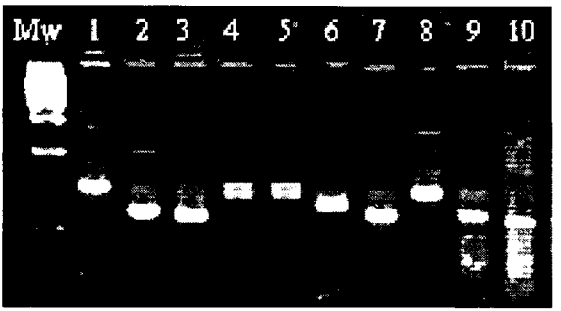

Fig.e 2. Detection of polymorphism using the nc 009 SSR marker

The data matrix was prepared using a total of 73 polymorphic fragments from 12 primer pairs, equivalent to 6.0 fragments/primer.

Table 2
Fragment numbers and PIC values expressing the polymorphism index content of gene-linked microsatellite (SSR) markers

SSR primer	Linked gene locus	Chromosomal localisation	No. of polymorphic fragments	PIC value
phi 095	*p1*	1.03	0	0.00
umc 1057	*ckol*	3.02	–	–
nc 004	*adh2*	4.03	–	–
phi 096	*zp1*	4.04	0	0.00
nc 007	*oph2*	5.01	nondescript differences	–
umc 1654	*rps15*	5.03	2	0.57
phi 85	*gln4*	5.06	4	0.54
y1SSR	*y1*	6.01	4	0.69
umc 1178	*mir2*	6.02	1	0.04
nc 009	*pl1*	6.04	12	0.88
phi 070	*mlg3*	6.07	nondescript differences	–
umc 1066	*op2*	7.01	5	0.80
umc 1741	*rps28*	8.03	5	0.54
umc 1069	*gst1*	8.08	7	0.82
phi 033	*sh1*	9.01	5	0.70
phi 061	*wx1*	9.03	–	–
phi 032	*sus1*	9.04	4	0.70
phi 062	*mgs1*	10.04	10	0.88
phi 084	*nac1*	10.04	13	0.90
*	*wx**	*	–	–
Average values of polymorphic loci			6.0	0.67

*Chromosomal localisation and sequence of the marker are unknown

Correlation analysis on the fragment patterns of SSR markers and the starch content of the lines

When evaluating the results obtained with genetic markers, the fragment patterns of three SSR markers suggested a correlation with the starch content of the maize lines. These were the primer pairs y1 SSR, umc 1069 and phi 062.

These results are only of a preliminary nature, however, as the incorporation of starch is probably regulated by several genes, and the studies suggest it is also influenced by several environmental factors. It also appears likely that the bioethanol yield is determined not only by the starch content, but also by other parameters. The chemical structure and fermentability of the starch molecules may also have a great influence on bioethanol quantity in the course of industrial production. Further research should thus be expanded to include investigations into the structural and fermentability traits of starch molecules, including the characterisation of these traits using genetic markers.

Conclusions

In summary it can be stated that SSR markers proved to be extremely efficient in detecting genetic polymorphism between the maize lines, and could thus be useful tools for characterising traits such as starch content by means of genetic markers. It should be emphasised, however, that environmental effects appear to play an important role in the development of the starch content, so further studies will be required to determine what factors influence bioethanol yield. It is suggested that the chemical structure and fermentability of the starch molecules, which are not greatly affected by environmental factors, should be subjected to both laboratory tests and to genetic marker analysis

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DETECTION OF THE 1RS CHROMOSOME ARM IN MARTONVÁSÁR WHEAT GENOTYPES CONTAINING 1BL.1RS OR 1AL.1RS TRANSLOCATIONS USING SSR AND STS MARKERS

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Several molecular markers have been reported for the detection of the 1RS chromosome arm. The aim of the present experiments was to study the reliability and reproducibility of six molecular markers specific to the 1RS rye chromosome (GPI, Bmac213, 5S, IAG95, SCM9 and RMS13) in distinguishing between wheat genotypes with and without the 1BL.1RS or 1AL.1RS translocations. In the course of the analysis, PCR products of the expected size were obtained with all the markers, which were found to give a reliable indication of the presence of the 1RS chromosome arm in the wheat genome.

Key words: 1RS chromosome arm, marker-assisted selection, molecular markers, sequence tagged site (STS), simple sequence repeat (SSR), winter wheat

Introduction

Rye is one of the most important cereals in the cool climate of northern Europe. Rye has numerous favourable traits: it is generally resistant to diseases, tolerant of low temperatures and able to realize relatively high grain yields on less fertile soils. One of the most common alien translocations in bread wheat varieties (1BL.1RS) originates from rye variety Petkus. Other translocations containing 1RS chromosome arms have also been produced: the 1RS arm of the 1AL.1RS translocation in the wheat cultivar Amigo can be traced back to the rye cultivar Insave (Zeller and Fuchs, 1983), while the 1DL.1RS translocation in the wheat cultivar Gabo is derived from the rye cultivar Imperial (Shepherd, 1973). Among the resistance genes found on the 1RS chromosome arm (*Lr26, Sr31, Yr9* and *Pm8*), only the wheat stem rust resistance gene *Sr31* is still effective (Bedő et al., 1993), though Pretorius et al. (2000) detected virulence to *Sr31* in Uganda, Africa. This is now spreading rapidly and endangering wheat

production all over the world. This can be attributed to the fact that all the 1BL.1RS translocations found in wheat varieties are derived from the rye variety Petkus, which means that there is no allelic variability. It would thus be advisable to replace the 1RS from Petkus by 1RS chromosomes from other resistant rye genotypes and also to select prospective wheat varieties that do not carry the 1BL.1RS translocation, so breeders need a rapid, reliable method for the detection of the 1RS chromosome arm in prospective varieties.

Genomic *in situ* hybridisation (GISH) is a high precision technique ideally suited for the detection of alien chromatin, such as the 1RS chromosome arm, in the genetic background of wheat (Molnár-Láng et al., 1996a; Kőszegi et al., 2000; Nagy and Molnár-Láng, 2000; Szakács et al., 2004). However, it is rather time-consuming and labour-intensive.

The use of PCR-based molecular markers is considerably faster. To date, numerous markers have been mapped on the chromosomes of the rye genome (Börner and Korzun, 1998; Khlestkina et al., 2004), including the 1RS chromosome arm (Nagy et al., 2003; Nagy and Lelley, 2003), thus allowing this arm to be rapidly detected in plant materials. The use of PCR-based markers allows large numbers of plant samples to be treated simultaneously, so the application of 1RS-specific SSR and STS markers provides a rapid and efficient method for the detection of the 1RS chromosome arm and of other traits (e.g. rust resistance genes) during the development of new wheat varieties (Gál et al., 2007; Hudcovicová et al., 2008). If breeding is to be successful it is essential to know the genome composition of varieties and advanced lines, so the detection of the 1RS chromosome arm is extremely important.

The aim of the present experiments was to study the reliability and reproducibility of six molecular markers specific to the 1RS rye chromosome arm (GPI, Bmac213, 5S, IAG95, SCM9 and RMS13) in order to detect the 1RS chromosome arm in rye varieties of different origin and to distinguish wheat genotypes with and without the 1BL.1RS or 1AL.1RS translocations.

Materials and methods

Rye varieties of different origin and Martonvásár wheat genotypes with or without the 1BL.1RS or 1AL.1RS translocations were used in the experiments (Table 1).

Six different 1RS-specific PCR-based molecular markers [GPI (van Capenhout, 1997), Bmac213 (Ramsay et al., 2000), 5S (Koebner, 1995), IAG95 (Mohler et al., 2001), SCM9 (Saal and Wricke, 1999) and RMS13 (Korzun, unpublished)] were used to detect the 1RS chromosome arm in rye and wheat genotypes. The PCR reaction was performed on an Eppendorf Mastercycler in a total reaction volume of 15 µl containing 40 µg genomic DNA, 1 × PCR buffer, 0.6 U Taq polymerase (Promega), 0.3 µl each of forward and reverse primers and 100 µM dNTPs. The PCR product was electrophoresed together with a size marker (Sigma or Fermentas) on 1% or 2.5% agarose gel with 90V or 280V voltage for approx. 60 minutes. The bands were visualised by ethidium bromide staining. Images were taken with the help of a Syngene G Box gel documentation system (Syngene, Cambridge, UK). The experiments were carried out in three replications.

Table 1
List of wheat and rye genotypes used for PCR analysis

Name	Translocation	Source
Chinese Spring (wheat, China)	–	Gene Bank, ARI, HAS, Martonvásár, Hungary
Mv9 kr1 (wheat, Hungary)	–	Martonvásár (Molnár-Láng et al., 1996b)
Mv Gorsium (wheat, Hungary)	1B/1R	Martonvásár
Mv Walzer (wheat, Hungary)	1B/1R	Martonvásár
Mv Magdaléna (wheat, Hungary)	1B/1R	Martonvásár
Mv Táltos (wheat, Hungary)	1A/1R	Martonvásár
Mv 07-03 (wheat, Hungary)	1A/1R	Martonvásár
Mv Dalma (wheat, Hungary)	1A/1R	Martonvásár
Mv 12-04 (wheat, Hungary)	1A/1R	Martonvásár
Sampo (rye, Finland)	–	USDA ARS, NSGC (Aberdeen, Idaho, USA)
Mansilla (rye, Spain)	–	USDA ARS
Dominant (rye, The Netherlands)	–	USDA ARS
Forrajero Klein (rye, Argentina)	–	USDA ARS
Beaulieu (rye, France)	–	USDA ARS
Porto (rye, Portugal)	–	USDA ARS
Jogeva 112 (rye, Estonia)	–	USDA ARS
Otello (rye, Sweden)	–	USDA ARS
Haru 4 (rye, Japan)	–	USDA ARS
Imperial (rye)	–	Driscoll and Sears, 1971

Results and discussion

Three STS (GPI, 5S, IAG95) and three SSR (Bmac213, SCM9, RMS13) markers were tested for the detection of the 1RS chromosome arm in various wheat and rye genotypes. All six molecular markers were found to be suitable for the detection of this chromosome arm and for distinguishing between wheat genotypes with and without the 1RS rye chromosome arm. PCR products of the expected size were obtained with all six markers.

The STS marker GPI amplified an approx. 270 bp product on wheat genotypes carrying the 1BL.1RS or 1AL.1RS translocations and on the rye varieties examined, while no PCR product was observed for the negative control wheats Chinese Spring and Mv9 kr1, which do not contain the 1RS chromosome arm (Figs. 1 and 2).

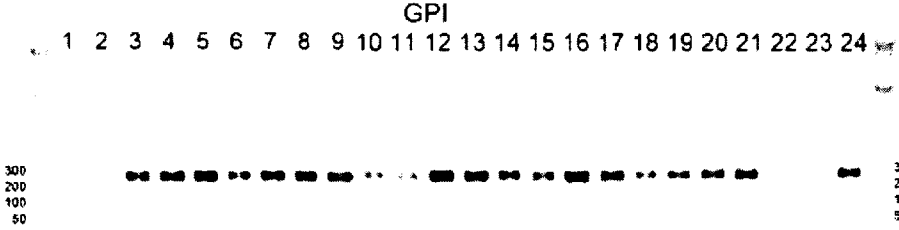

Fig. 1. Molecular marker analysis of wheat genotypes Chinese Spring (1 and 22), Mv9 kr1 (2 and 23) and Mv Magdaléna (3 and 24) and rye varieties Sampo (4, 5), Mansilla (6, 7), Dominant (8, 9), Forrajero Klein (10, 11), Beaulieu (12, 13), Porto (14, 15), Jogeva 112 (16, 17), Otello (18, 19) and Haru 4 (20, 21) on 1% agarose gel with the STS marker GPI

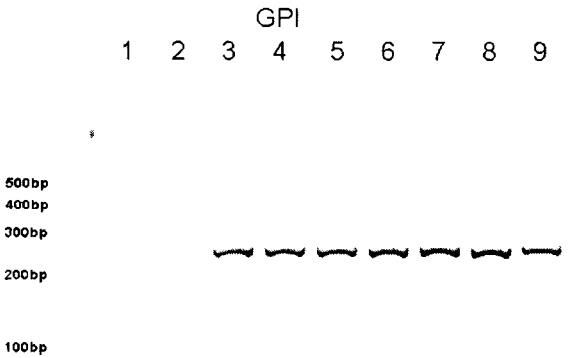

Fig. 2. Molecular marker analysis of wheat genotypes Chinese Spring (1), Mv9 kr1(2), Mv Gorsium (3), Mv Walzer (4), Mv Magdaléna (5), Mv Táltos (6), Mv 07-03 (7), Mv Dalma (8) and Mv 12-04 (9) on 2.5% agarose gel with the STS marker GPI

Similar results were found using the 1RS-specific SSR marker Bmac213. An approx. 500 bp DNA segment was observed for all the rye varieties tested and for all the wheat genotypes containing the 1BL.1RS or 1AL.1RS translocations, which was absent in wheats Chinese Spring and Mv9 kr1 (Figs. 3 and 4).

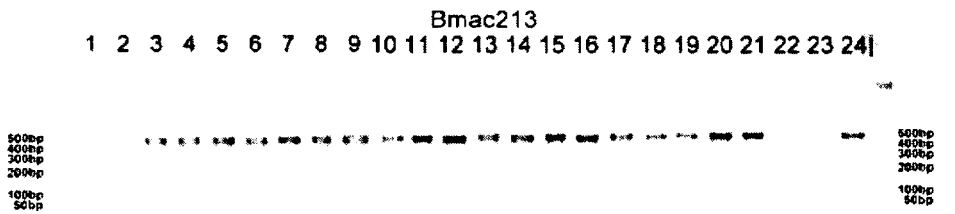

Fig. 3. Molecular marker analysis of wheat genotypes Chinese Spring (1 and 22), Mv9 kr1 (2 and 23) and Mv Magdaléna (3 and 24) and rye varieties Sampo (4, 5), Mansilla (6, 7), Dominant (8, 9), Forrajero Klein (10, 11), Beaulieu (12, 13), Porto (14, 15), Jogeva 112 (16, 17), Otello (18, 19) and Haru 4 (20, 21) on 1% agarose gel with the SSR marker Bmac213

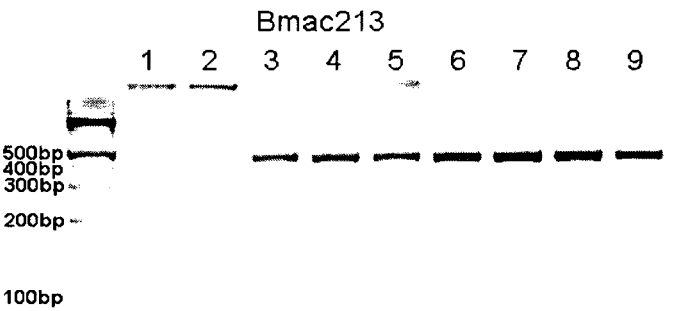

Fig. 4. Molecular marker analysis of wheat genotypes Chinese Spring (1), Mv9 kr1 (2), Mv Gorsium (3), Mv Walzer (4), Mv Magdaléna (5), Mv Táltos (6), Mv 07-03 (7), Mv Dalma (8) and Mv 12-04 (9) on 2.5% agarose gel with the SSR marker Bmac213

STS markers 5S and IAG95, and SSR marker SCM9 gave an approx. 100 bp diagnostic PCR product for the rye varieties and for all the wheat genotypes carrying the 1BL.1RS or 1AL.1RS translocations, while no PCR products characteristic of the 1RS chromosome arm were observed for the negative controls, Chinese Spring and Mv9 kr1 (Figs. 5, 6, 7 and 8). The STS marker IAG95 amplified a weaker band, 300 bp in length, which was observed for all wheat genotypes containing the 1RS chromosome arm (Fig. 7). Two additional weak bands were obtained on the agarose gels with the SSR marker SCM9 (Fig. 8). One of these weak bands was diagnostic between wheat genotypes carrying the 1BL.1RS or the 1AL.1RS translocations (Fig. 8; Weng et al., 2007). Weng et al. (2007) observed a 207 bp band in all wheat lines containing the 1BL.1RS translocation, while a 228 bp band was present in all wheat lines carrying the 1AL.1RS translocation. The diagnostic fragment observed in the present study appears to be the same length as that obtained by Weng et al. (2007).

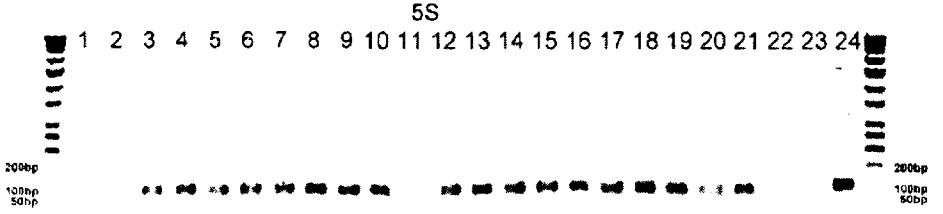

Fig. 5. Molecular marker analysis of wheat genotypes Chinese Spring (1 and 22), Mv9 kr1 (2 and 23) and Mv Magdaléna (3 and 24) and rye varieties Sampo (4, 5), Mansilla (6, 7), Dominant (8, 9), Forrajero Klein (10, 11), Beaulieu (12, 13), Porto (14, 15), Jogeva 112 (16, 17), Otello (18, 19) and Haru 4 (20, 21) on 1% agarose gel with the STS marker 5S

Fig. 6. Molecular marker analysis of wheat genotypes Chinese Spring (1), Mv9 kr1 (2), Mv Gorsium (3), Mv Walzer (4), Mv Magdaléna (5), Mv Táltos (6), Mv 07-03 (7), Mv Dalma (8) and Mv 12-04 (9) on 2.5% agarose gel with the STS marker 5S

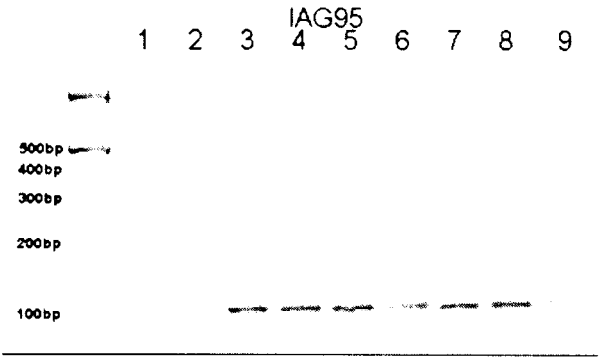

Fig. 7. Molecular marker analysis of wheat genotypes Chinese Spring (1), Mv9 kr1 (2), Mv Gorsium (3), Mv Walzer (4), Mv Magdaléna (5), Mv Táltos (6), Mv 07-03 (7), Mv Dalma (8) and Mv 12-04 (9) on 2.5% agarose gel with the STS marker IAG95

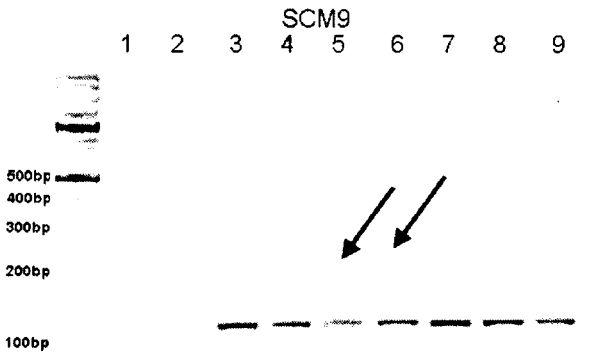

Fig. 8. Molecular marker analysis of wheat genotypes Chinese Spring (1), Mv9 kr1 (2), Mv Gorsium (3), Mv Walzer (4), Mv Magdaléna (5), Mv Táltos (6), Mv 07-03 (7), Mv Dalma (8) and Mv 12-04 (9) on 2.5% agarose gel with the SSR marker SCM9. Diagnostic bands between wheat genotypes containing 1BL.1RS or 1AL.1RS translocations are indicated with arrows

SSR marker RMS13 amplified two main bands diagnostic for the 1RS chromosome arm, which are approx. 175 and 150 bp long. These bands could only be observed for wheat genotypes containing the 1RS chromosome arm (Fig. 9).

The 1RS-specific molecular markers GPI, Bmac213, 5S, IAG95, SCM9 and RMS13 were thus capable of reliably detecting the presence of the 1RS chromosome arm in the wheat genome. The negative results obtained for the control wheat genotypes (without 1BL.1RS or 1AL.1RS translocations) confirmed the reliability of the markers. Using these markers in three replications proved to be very reliable in separating plants with or without the 1RS chromosome arm. The SSR marker SCM9 showed polymorphisms between the 1BL.1RS and 1AL.1RS translocations due to their different origin (Weng et al., 2007). It can be concluded that SCM9 is a fast and reliable marker to identify and differentiate 1AL.1RS and 1BL.1RS translocations. All the markers tested

could thus be used in breeding for the marker-assisted selection of genotypes with or without the 1RS chromosome arm. Marker-assisted selection facilitates the detailed genetic analysis of large numbers of prospective varieties in wheat breeding. In order to avoid genetic uniformity it is important to trace the occurrence of the 1BL.1RS and 1AL.1RS translocations in breeding materials.

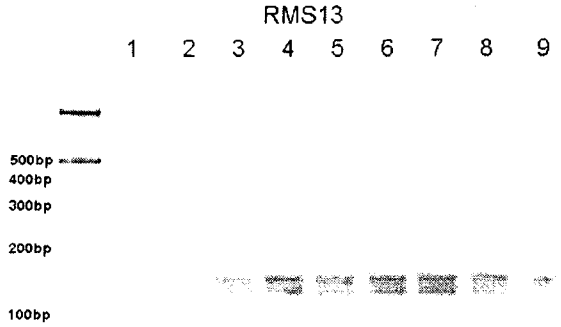

Fig. 9. Molecular marker analysis of wheat genotypes Chinese Spring (1), Mv9 kr1 (2), Mv Gorsium (3), Mv Walzer (4), Mv Magdaléna (5), Mv Táltos (6), Mv 07-03 (7), Mv Dalma (8) and Mv 12-04 (9) on 2.5% agarose gel with the SSR marker RMS13

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COMBINING ABILITY AND GENE ACTION STUDIES FOR YIELD-CONTRIBUTING TRAITS IN CROSSES INVOLVING WINTER AND SPRING WHEAT GENOTYPES

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The success of winter × spring wheat hybridization programmes depends upon the ability of the genotypes of these two physiologically distinct ecotypes to combine well with each other. Hence the present investigation was undertaken to study the combining ability and nature of gene action for various morpho-physiological and yield-contributing traits in crosses involving winter and spring wheat genotypes. Five elite and diverse genotypes each of winter and spring wheat ecotypes and their F_1 (spring × spring, winter × winter and winter × spring) hybrids, generated in a diallel mating design excluding reciprocals, were evaluated in a random block design with three replications. Considerable variability was observed among the spring and winter wheat genotypes for all the traits under study. Furthermore, these traits were highly influenced by the winter and spring wheat genetic backgrounds, resulting in significant differences between the spring × spring, winter × winter and winter × spring wheat hybrids for some of the traits. The winter × spring wheat hybrids were observed to be the best with respect to yield-contributing traits. On the basis of GCA effects, the spring wheat parents HPW 42, HPW 89, HW 3024, PW 552 and UP 2418 and the winter wheat parents Saptdhara, VWFW 452, W 10 and WW 24 were found to be good combiners for the majority of traits. These spring and winter wheat parents could be effectively utilized in future hybridization programmes for wheat improvement. Superior hybrid combinations for one or more traits were identified, all of which involved at least one good general combiner for one or more traits in their parentage, and can thus be exploited in successive generations to develop potential recombinants through various breeding strategies. Genetic studies revealed the preponderance of additive gene action for days to flowering, days to maturity and harvest index, and non-additive gene action for the remaining six traits.

Key words: genetic variation, winter × spring wheat hybridization, combining ability, gene action

Introduction

A spectacular increase in wheat production and productivity has been achieved in India since the times of the 'Green Revolution' with the introduction and exploitation of the Norin 10 dwarfing genes *Rht 1* and *Rht 2* to develop

high-yielding, semi-dwarf varieties of wheat in the mid-60s. However, at present, a plateau appears to have been reached in wheat production, productivity and per capita availability, due to static acreage and a continuous increase in the population, which will necessitate another breakthrough to improve the yield potential of bread wheat. To accomplish this, one breeding approach is to utilize the physiologically distinct winter wheat gene-pool, grown only in the dry and wet temperate high hill regions of the North-Western Himalayas, for the amelioration of the spring wheat cultivars predominantly grown. These two groups have remained almost independent of each other due to their entirely different ecological requirements for development (Akerman and Mackey, 1949). Important attributes of winter wheat ecotypes, such as drought and cold tolerance (Rajaram and Skovmand, 1977), additional sources of resistance to stripe rust (Upadhyay and Kumar, 1975), leaf rust (Bartos et al., 1969), powdery mildew (Chaudhary and Kapoor, 1992) and *Septoria* (Kochumadhavan et al., 1988), and other useful traits like better grain quality, profuse tillering, large and highly fertile square heads, good crown root system and enhanced yield (Pinthus, 1967; Grant and McKenzie, 1970) can be introgressed into spring wheat following winter × spring wheat hybridization programmes. The success of such a breeding approach depends upon the ability of these two ecotypes to combine well with each other, so as to exploit the variability present in them in order to evolve superior recombinants suited for cultivation under varying ecological conditions, but very little information is available in the literature. Keeping this in view, the present investigation was undertaken to study the genetic variability among genotypes of winter and spring ecotypes of wheat and the combining ability and nature of gene action governing various morpho-physiological and yield-contributing traits in crosses involving winter and spring wheat genotypes.

Materials and methods

The initial plant material consisted of five elite and diverse genotypes each of spring wheat (HPW 42, HPW 89, HW 3024, PW 552 and UP 2418) and winter wheat (Saptdhara, Sentry, VWFW 452, W 10 and WW 24). For the success of winter × spring wheat hybridization programmes, the flowering period of these two ecotypes must synchronize for a long spell. The vernalization requirement of winter wheat genotypes at Palampur was fulfilled by subjecting germinating seeds to chilling treatment at 4 ± 0.5°C on wet filter paper for 30 days prior to cultivation in the field. The crosses were made in a diallel mating design excluding reciprocals (Griffing's Method II, Half-diallel) to generate 45 F_1 (spring × spring, winter × winter and winter × spring) hybrids, all of which were raised along with the 10 parents in a randomized block design (RBD) with three replications. The germinating seeds of the winter wheat parents and their F_1s were vernalized by subjecting them to chilling treatment at 4° ± 0.5°C for 30 days before sowing in the field.

Replication-wise data, on the basis of five randomly sampled competitive plants, were recorded for seven different traits, namely plant height (cm), tillers per plant, spikelets per spike, grains per spike, grain yield per plant (g), 1000-grain weight (g) and harvest index (%), while the observations for days to 50% flowering and days to 90% maturity were recorded on a plot basis. The data were subjected to analysis of variance (RBD) to determine significant differences within and between the spring and winter wheat parents, as well as within and between the spring × spring, winter × winter and winter × spring hybrids. The least significant difference (LSD)

between and within groups was determined as follows:

$$\text{LSD between groups} = \sqrt{2EMS/(r \times g)}$$
$$\text{LSD within groups} = \sqrt{2EMS/r},$$

where EMS is the error mean sum of squares, r is the number of replications and g is the number of genotypes.

The mean values of each genotype over three replications for the various traits were used to analyse the data using Griffing's (1956) approach to study the general combining ability (GCA) effects of the winter and spring wheat parents, the specific combining ability (SCA) effects of the crosses and the genetic components governing the inheritance of the traits studied. The fixed effect model (Model I) was used to calculate the genetic variances/components. The relative importance of GCA and SCA, which demonstrates the significance of additive versus non-additive genetic variances, was calculated from the ratio $2\sigma^2g/(2\sigma^2g + \sigma^2s)$ given by Baker (1978) for a fixed effect model, where σ^2g is the variance due to GCA and σ^2s the variance due to SCA. If this ratio is ≥0.50, it indicates the preponderance of additive gene action in the inheritance of the traits, whereas the reverse is true for the non-additive/dominant gene action.

Results and discussion

The analysis of variance showed significant differences among the genotypes (parents and F_1s) for all the yield-contributing parameters (Table 1), indicating enormous genetic variability among the genotypes for all the traits under study. Further splitting the sum of squares due to genotypes into sum of squares between groups and within groups also showed significant differences between and within groups for all the traits studied. Within the spring and winter wheat parents, significant differences were observed with respect to all the traits except tillers per plant in spring wheats and tillers per plant and grain yield per plant in winter wheat genotypes. Significant differences were observed within the spring × spring and winter × spring wheat F_1 hybrids for all the traits except tillers per plant. The differences were highly significant for all the parameters studied within the winter × winter wheat crosses.

Table 1
Analysis of variance for different yield-contributing traits in wheat

Source of variation	d.f.	Mean squares								
		1	2	3	4	5	6	7	8	9
Replication	2	3.86	2.21	0.81	0.16	1.99	9.31	0.70	0.02	3.57
Genotype	54	226.34*	406.02*	179.35*	1.11*	18.07*	315.64*	11.50*	77.59*	52.27*
Genotypes^{+}	4	1425.39*	3152.6*	942.25*	5.02*	104.74*	1412.74*	63.17*	449.59*	52.56*
Genotypes^{++}	50	130.42*	186.28*	118.31*	0.80*	11.14*	227.87*	7.34*	47.83*	52.24*
Spring wheat parents	4	67.73*	153.83*	264.14*	0.66	10.81*	32.25*	3.33*	51.72*	14.80
Winter wheat parents	4	506.77*	681.07*	174.96*	0.26	11.37*	323.26*	1.13	29.34*	179.95*
Spring × spring wheats	9	16.55	151.04*	47.87*	0.31	16.92*	232.50*	10.88*	37.36*	20.58*
Winter × winter wheats	9	91.32*	84.26*	79.19*	1.61*	9.39*	316.41*	11.00*	27.02*	42.49*
Winter × spring wheats	24	135.50*	160.69*	125.65*	0.80*	9.65*	209.63*	6.35*	62.00*	52.73*
Error	108	11.61	8.34	7.73	0.47	1.35	12.68	1.30	6.19	8.79

1: Days to flowering; 2: Days to maturity; 3: Plant height, 4: Tillers/plant; 5: Spikelets/spike; 6: Grains/ spike; 7: Grain yield/plant; 8: 1000-grain weight; 9: Harvest index; $^{+}$: between groups, $^{++}$: within groups; * Significant at $P \leq 0.05$

Among the different groups, the spring wheat genotypes were early maturing, with greater plant height, number of tillers per plant, spikelets per spike, grains per spike, grain yield per plant, 1000-grain weight and harvest index under Palampur conditions. To study the genetic variability among winter and spring wheat genotypes in respect of grain and green fodder yield, Chaudhary et al. (1994) evaluated seven exotic winter wheat varieties, along with two spring wheat varieties as standard checks in the dry, temperate region of the north-west Himalayas and found that the winter wheat varieties outyielded the spring wheat controls even after obtaining a green fodder cutting from the former.

Complementary interactions among the winter and spring wheat parents resulted in the highest number of grains per spike and grain yield per plant in winter × spring wheat hybrids in comparison to the spring × spring and winter × winter wheat hybrids (Table 2). Among the F_1 hybrids, the winter × spring and winter × winter wheat hybrids had significantly more tillers per plant and spikelets per spike and took significantly longer to attain maturity than the spring × spring wheat hybrids. The winter × spring and winter × winter wheat hybrids performed at par for these traits. Significant differences were observed between the spring × spring, winter × winter and winter × spring wheat hybrids for days to flowering. The spring × spring wheat crosses had the lowest number of days to flowering, followed by winter × spring and winter × winter wheat crosses. However, the differences were non-significant between the parents for days to flowering, days to maturity, plant height, spikelets per spike, 1000-grain weight and harvest index, suggesting the interactive influence of spring and winter wheat genotypes on various yield-contributing traits, resulting in significant differences between the spring × spring, winter × winter and winter × spring wheat hybrids for some of the traits studied. Considering the enormous genetic variability among the winter and spring wheat genotypes, Chaudhary (1997) suggested the use of winter wheats for the improvement of spring wheat genotypes through hybridization followed by selection for desirable traits such as early maturity, semi-dwarf height, high number of grains and spikelets per spike, profuse tillering, higher yield and high harvest index.

The analysis of variance for combining ability (Table 3) showed the significance of GCA and SCA for all nine traits, indicating the importance of both additive and non-additive gene actions in the inheritance of these traits.

The estimates of GCA for the parents are given in Table 4. Overall, on the basis of GCA effects, the best general combiners were the spring wheat parents HPW 42 for days to 50% flowering, days to maturity and harvest index, HPW 89 for days to 50% flowering, days to maturity, plant height, grains per spike, grain yield per plant and 1000-grain weight, HW 3024 for days to 50% flowering, days to maturity and 1000-grain weight, PW 552 for plant height, spikelets per spike, grains per spike, grain yield per plant, 1000-grain weight and harvest index, and UP 2418 for 1000-grain weight. Among the winter wheat parents, Saptdhara was identified as a good general combiner for plant height,

spikelets per spike and tillers per plant, VWFW 452 for spikelets per spike, grains per spike and harvest index, W 10 for days to 50% flowering, grains per spike, grain yield per plant and harvest index, and WW 24 for plant height. The winter wheat parent Sentry was observed to be a poor combiner for the majority of the traits studied. Thus, the spring wheat parents, HPW 42, HPW 89, HW 3024, PW 552 and UP 2418 and the winter wheat parents, Saptdhara, VWFW 452, W 10 and WW 24 could be effectively utilized in future hybridization programmes for wheat improvement.

On the basis of SCA effects, the majority of the cross combinations were identified as superior hybrid combinations for one or more traits (data not shown). The best combinations (for at least four parameters) are given in Table 5. All these combinations involved at least one good general combiner for one or more traits in their parentage and could thus be exploited in successive generations for developing superior recombinants.

Table 2
Average frequencies of yield-contributing parameters in different groups of wheat parents and their F_1 hybrids

Genotypic group	1	2	3	4	5	6	7	8	9
Spring wheat (S)	123.7	159.3*	74.4*	3.0*	16.6*	51.7*	5.3*	49.0*	43.1*
Winter wheat (W)	125.8	172.1	63.0	2.2	14.3	33.3	2.4	45.7	40.8
CD (5%)	2.46	2.09	2.01	0.50	0.84	2.58	0.82	1.80	2.14
S × S	121.6*	157.6*	79.8*	2.9	16.9	47.9	5.6	56.9*	45.3
W × W	139.3*	180.4	76.1	3.4*	19.5*	47.6	5.6	48.5	44.0
CD (5%)	1.74	1.48	1.42	0.35	0.59	1.82	0.58	1.27	1.52
S × S	121.6*	157.6*	79.8	2.9	16.9	47.9	5.6	56.9*	45.3
W × S	147.1	177.0	79.5	3.3*	19.0*	53.9*	6.8*	52.6	45.0
CD (5%)	1.46	1.24	1.19	0.29	0.50	1.52	0.49	1.06	1.27
W × W	139.3*	180.4	76.1	3.4	19.5	47.6	5.6	48.5	44.0
W × S	147.1	177.0*	79.5*	3.3	19.0	53.9*	6.8*	52.6*	45.0
CD (5%)	1.46	1.24	1.19	0.29	0.50	1.52	0.49	1.06	1.27

1: Days to flowering; 2: Days to maturity; 3: Plant height, 4: Tillers/plant; 5: Spikelets/spike; 6: Grains/spike; 7: Grain yield/plant; 8: 1000-grain weight; 9: Harvest index; *Significant at $P \leq 0.05$

Table 3
Analysis of variance for combining ability for different yield-contributing traits

Source of variation	d.f.	Mean squares								
		1	2	3	4	5	6	7	8	9
GCA	9	328.75*	506.03*	144.80*	0.31*	11.74*	189.95*	5.04*	57.40*	57.05*
SCA	45	24.78*	61.21*	42.78*	0.38*	4.88*	88.26*	3.58*	19.56*	9.50*
Error	108	3.87	2.78	2.58	0.16	0.45	4.23	0.43	2.06	2.93
$2\sigma^2g/2(\sigma^2g+\sigma^2s)$		0.72	0.59	0.37	0.08	0.30	0.27	0.19	0.35	0.58

1: Days to flowering; 2: Days to maturity; 3: Plant height, 4: Tillers/plant; 5: Spikelets/spike; 6: Grains/spike; 7: Grain yield/plant; 8: 1000-grain weight; 9: Harvest index; GCA: General combining ability; SCA: Specific combining ability; * Significant at $P \leq 0.05$

Table 4
Estimates of general combining ability (GCA) effects of the parents for different yield-contributing traits

Genotypes	1	2	3	4	5	6	7	8	9
					Spring wheat				
HPW 42	−3.83*	−7.96*	0.60	−0.15	−0.34	1.08	−0.30	0.31	2.08*
HPW 89	−2.27*	−4.93*	4.29*	−0.09	−0.01	1.73*	0.56*	2.08*	−0.03
HW 3024	−5.91*	−5.76*	0.66	0.07	−1.32*	−3.54*	−0.24	2.29*	−0.59
PW 552	0.59	0.96*	3.97*	0.18	1.66*	8.02*	1.15*	1.45*	1.60*
UP 2418	−4.13*	−6.62*	−1.72*	−0.13	−1.22*	0.63	0.08	2.18*	−0.09
					Winter wheat				
Saptdhara	12.56*	12.96*	2.45*	0.26*	1.15*	−2.75*	−0.03	−2.17*	−4.37*
Sentry	3.51*	5.32*	−4.06*	−0.17	0.13	−4.57*	−1.14*	−3.98*	−2.22*
VWFW 452	−0.22	1.32*	−6.85*	0.02	0.88*	1.99*	0.18	0.07	1.95*
W 10	−1.61*	0.79	−0.39	0.17	−0.18	2.26*	0.43*	0.19	2.62*
WW24	1.31*	3.91*	1.05*	−0.15	−0.76*	−4.85*	−0.69*	−2.44*	−0.94*

1: Days to flowering; 2: Days to maturity; 3: Plant height, 4: Tillers/plant; 5: Spikelets/spike; 6: Grains/spike; 7: Grain yield/plant; 8: 1000-grain weight; 9: Harvest index; * Significant at $P \leq 0.05$

Table 5
Superior combinations identified on the basis of their specific combining ability effects

Combinations	1	2	3	4	5	6	7	8	9
					Winter × winter wheat				
Saptdhara × VWFW 452	−0.58	−9.63*	2.53	1.27*	1.24*	−5.57*	2.49*	1.23	0.54
Saptdhara × W 10	1.47	−1.77	5.94*	0.58	2.67*.	18.80*	1.37*	−1.26	−2.16
Sentry × W 10	6.86*	6.87*	3.25*	0.68	1.79*	−5.26*	1.74*	3.61*	2.63
VWFW 452 × W10	−1.08	−4.13*	8.30*	0.69	3.07*	9.28*	1.06	−1.91	−1.78
					Winter × spring wheat				
Saptdhara × HPW 42	2.36	9.98*	9.55*	0.20	1.85*	9.81*	3.08*	10.46*	7.21*
Saptdhara × UP 2418	−1.00	4.32*	10.24*	0.07	0.50	10.36*	1.46*	−0.45	5.85*
Sentry × HPW 42	1.75	5.62*	6.93*	1.30	2.07*	7.32*	1.94*	−2.74*	−2.14
Sentry × HPW 89	−0.14	4.26*	9.14*	0.80*	2.51*	3.61	3.19*	1.23	−0.89
VWFW452 × PW552	1.06	1.04	3.38*	0.58	1.09	7.63*	1.64*	4.47*	−0.23
VWFW452 × UP2418	4.78*	9.29*	−0.63	0.75	1.20	7.54*	2.01*	4.24*	2.76
WW 24 × HPW 42	1.28	5.70*	2.93*	0.55	1.62*	5.38*	1.87*	6.22*	0.68
WW 24 × UP 2418	−0.08	2.70	8.28*	0.29	2.74*	8.19*	2.19*	7.75*	1.49

1: Days to flowering; 2: Days to maturity; 3: Plant height, 4: Tillers/plant; 5: Spikelets/spike; 6: Grains/spike; 7: Grain yield/plant; 8: 1000-grain weight; 9: Harvest index; * Significant at $P \leq 0.05$

The relative magnitude of GCA and SCA (Table 3) indicated a predominant role for SCA for six traits (plant height, tillers per plant, spikelets per spike, grains per spike, grain yield per plant and 1000-grain weight) and for GCA in days to 50% flowering, days to maturity and harvest index, indicating non-additive gene action in the inheritance of the former six traits and additive gene action in the latter three. These findings indicated that the traits days to flowering, days to maturity and harvest index were fixable and that effective selection could be made for these traits during segregating generations, while the other six traits were not fixable in the segregating generations, so selection for these traits in earlier generations is not effective and must be postponed till later generations.

In all, the present investigation demonstrated the existence of considerable genetic variability among the winter and spring wheat genotypes and their F_1 hybrids for the majority of yield-contributing traits, but these were also highly influenced by the winter and spring wheat genetic backgrounds and their interactions, resulting in significant differences between the spring × spring, winter × winter and winter × spring wheat hybrids for some of the traits under study. The spring and winter wheat genotypes and hybrid combinations identified on the basis of their combining ability effects have immense potential for use in future wheat improvement programmes to develop high-yielding lines of wheat. The genetic studies showed the preponderance of additive gene action for days to flowering, days to maturity and harvest index, and of non-additive gene action for the remaining six traits.

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SPOT BLOTCH AND TERMINAL HEAT STRESS TOLERANCE IN SOUTH ASIAN SPRING WHEAT GENOTYPES

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Terminal heat stress and spot blotch disease (caused by *Cochliobolus sativus*) are the most important stresses responsible for significant yield losses every year in warm South Asian plains. Both of these stresses are very severe in late planted wheat, which is common in rice–wheat and rice–rice–wheat cropping systems. The development of genotypes tolerant to both stresses might be very useful for increasing yield and reducing yield losses. Information is limited on how different genotypes respond to both stresses (individually and combined) and on the degree of tolerance present in South Asian wheat genotypes. The study was done to evaluate the tolerance of South Asian wheat genotypes to both stresses by comparing the stress factor susceptibility index (SFSI). Eleven diverse South Asian genotypes were evaluated under spot blotch stress (non-fungicide protected plots), heat stress (late planted and fungicide protected), both stresses (non-fungicide protected and late planted) and normal planting situations (fungicide protected and normal season planted) at Rampur, Chitwan, Nepal. Both stresses reduced the grain yield and thousand-kernel weight (TKW), but not other yield components, including grains/spike and spikelets/spike. Genotypes BL 1473, Gautam and NL971 were moderately to highly tolerant to both types of stress. Generally genotypes that are tolerant or resistant to spot blotch also showed tolerance to heat stress, suggesting a common physiological mechanism to combat both stresses in tolerant genotypes.

Key words: *Cochliobolus sativus* Sacc., foliar blight, late planting stress, stress factor susceptibility index

Introduction

For wheat, heat stress and spot blotch disease are two of the most important stresses in non-traditional, warm wheat-growing areas of the world, causing significant yield losses (Dubin and Rajaram, 1996; Duveiller and Gilchrist, 1994). Regions subject to both these stresses are classified by CIMMYT as Mega environment 5A. Spot blotch, caused by *Cochliobolus*

sativus (Ito and Kurbayzshi) Drechsler ex Dastur, puts 25 million hectares of land under pressure (Duveiller and Gilchrist, 1994), whereas terminal heat is a problem in at least 40% of the irrigated wheat growing areas of the world (Fischer and Byerlee, 1991). Both of these stresses cause loss of photosynthetically active leaves, premature leaf senescence, reduced grain filling, low kernel weight and severe grain yield reductions (Al-Khatib and Paulsen, 1990; Duveiller and Gilchrist, 1994; Joshi et al., 2007; Mercado et al., 2003).

Rice–wheat or rice–rice–wheat cropping systems cover large parts of the South Asian subcontinent (Hobbs et al., 1998), where wheat sowing is delayed due to the preceding rice crop and other management problems. Such late planting causes terminal heat stress (Sharma et al., 2008). Heat stress during GS3 (anthesis to maturity) mainly affects assimilate availability, translocation of photosynthates to the grain, and starch synthesis and deposition in the developing grain. High temperatures (>30°C) after anthesis have been reported to decrease the rate of grain filling (Randall and Moss, 1990). As the mean temperature increases from 12°–26°C during grain filling, grain weight is reduced at a rate of 4–8%/°C (Wiegand and Cuellar, 1981). The delayed sowing of wheat has also been found to increase spot blotch severity (Sharma and Duveiller, 2004). It is well known that both terminal heat stress (Gibson and Paulsen, 1999) and spot blotch (Rosyara et al., 2007; Sharma et al., 2008; Sharma and Duveiller, 2004) reduce grain yield due to reduced kernel weight.

Heat stress is already a problem in South Asian plains (Hobbs et al., 1998). Both the growing demand for food and global warming are expected to further push wheat crops to heat stress environments. There have also been reports on increased spot blotch severity and reduced thousand-kernel weight (TKW) in recent years (Sharma et al., 2007a). Consequently, breeding for heat stress tolerance and spot blotch resistance or tolerance are two important objectives of wheat improvement programmes targeting South Asian plains. Recently, breeding programmes in South Asia are using synthetic hexaploids (obtained by crossing tetraploid wheat and *Aegilops tauschii* Coss) as a resistant donor for spot blotch (Duveiller et al., 2005; Sharma et al., 2007b). Many synthetic hexaploids have proved useful as a source of tolerance to abiotic stresses (Gorham, 1990; Limin and Fowler, 1993) in addition to biotic stresses.

Genetic diversity for heat tolerance in cultivated wheat is well established (Al-Khatib and Paulsen, 1990; Lillemo et al., 2005; Reynolds, 1994). There is limited information on spot blotch tolerance; however, genotypes with a high level of resistance have been identified (Duveiller et al., 2005; Rosyara et al., 2007; Sharma et al., 2007b). None of the genotypes known to date has immunity to the disease, although some have a high level of resistance. In every situation, tolerance of the disease could be beneficial to reduce the risk of high yield losses. Studies are also limited on how South Asian wheat genotypes respond to combined spot blotch and heat stress and on the extent of variability for tolerance. Thus, the objective of this work was to study the effect of spot blotch and heat stress, individually and combined, on yield and yield components, and the variation in stress tolerance in South Asian genotypes.

Materials and methods

Field experiments were conducted at the Institute of Agriculture and Animal Science, Rampur, Chitwan, Nepal. The location is characterized by a warm, humid environment (27°37'N, 84°24'E, 228 metres above mean sea level) on the Gangetic Plains, where foliar blight epiphytotics occur every year, allowing field evaluation (Duveiller et al., 2005). The experiment was conducted in the wheat growing seasons of 2006 (November 2006 to May 2007) and 2007 (November 2007 to May 2008).

The design of the experiment was a randomized complete block design with strip-split-plot treatment arrangements. The vertical factor was spraying (two levels: no fungicidal spray vs. fungicidal spray to protect against the disease), whereas the horizontal factors were date of sowing (two levels: normal planting to avoid terminal heat stress and late planting to create high terminal heat stress) and genotypes. The arrangement of dates of sowing and genotypes was a split plot, where date of sowing was the main plot factor and genotype the subplot. The plot size was 3 m^2 (2 m long and 1.5 m wide). The trials were sown using the standard seeding rate of 120 kg ha^{-1}.

The genotypes included were Sonalika (RR21 = *II 54-8/An/3/Yt54/N10B//LR64*), Gautam (= BL 1887), BL 1473 (= *NL 297/NL 352*), BL 2662, BL 2217, BAW 343, Bhrikuti (= *CDO/COC/3/PLO//FURY/ANA*), Kanchan (= *UP 301/SUJATA*), UP 262 (= *S308/BAJIO 66*), NL971 (= *MRNG/BUC//BLO/PVN/3/PJB 81*) and WK 1204. The genotypes were developed for the South Asian region by the collaborative efforts of the CIMMYT International South Asia programme and the national agriculture research programmes of Nepal, India, Bangladesh and Pakistan.

Normal planting was done on 22 Nov and late planting on 30 Dec in both years. The optimal sowing time of wheat in this location is 20 Nov to 10 Dec. Late planting causes severe terminal heat stress, leading to severe yield losses (Hobbs et al., 1998). In disease-protected plots the fungicide 'Opus' (*Epoxiconazol*) 0.05% a.i. was sprayed at seven-day intervals, starting 50 days after sowing, until the complete senescence of the flag leaves. Spreader rows of Sonalika (a highly susceptible cultivar) were planted around each plot to generate high, uniform inoculum pressure. There was high uniform inoculum pressure in both years and no complementary artificial inoculation was required.

Fertilizers were applied at rates of 120 kg N, 60 kg P_2O_5 and 40 kg K_2O per hectare. The nitrogen dose was split, with 100 kg broadcast as basal fertilizer and 20 kg top-dressed at the active tillering stage. The plots were kept free of weeds by hand weeding. Irrigation was carried out as required by the crop in both years.

Three spot blotch disease scores (recorded as percentage of diseased leaf area on flag and penultimate leaf) were observed on ten randomly selected and tagged plants per plot at 5–7-day intervals. The resulting diseased leaf area (DLA) scores were used to calculate the Area Under the Disease Progress Curve (AUDPC) and AUDPC per day, following Duveiller et al. (2005).

Yield and yield components were measured as suggested by Sharma and Duveiller (2004). The whole plot was cut at ground level, sun dried to constant weight, weighed and threshed, and grain weight was recorded for the determination of biomass yield. The grain yield and thousand-kernel weight (TKW) were recorded on a constant moisture basis. The effective tiller number was counted in the two middle rows of each plot just before complete harvesting. Ten random spikes were taken per plot to measure spike length, spikelets per spike, florets per spike and grains per spike. The grain yield loss for each genotype was estimated as the percentage difference between normal (non-stressed) and stressed plots.

Heat stress tolerance was evaluated by comparing the grain yield and TKW from normal plots (normal planting, disease protected) with that of heat-stressed plots (late planting, disease protected) (Shpiler and Blum, 1986). Similarly, tolerance to spot blotch was evaluated by comparing the grain yield and TKW from normal plots (fungicide protected, timely planted) with that of spot blotch-stressed plots (without fungicide protection, normal planting). Tolerance to combined stress (spot blotch and heat stress) was evaluated by comparing normal plots (normal planting, disease protected) with plots receiving both stresses (late planting, disease non-

protected). For the numerical comparison of tolerance, the stress factor susceptibility index (SFSI) was calculated using the following formula, modified from Fischer and Maurer (1978):

$$SFSI = (1-Y/Y_p)/D$$

where Y = yield of a genotype in a stress environment (heat, spot blotch or combined), Y_p = yield under optimal planting conditions, D = stress intensity = $1 - X/X_p$, X = mean Y of all genotypes under stress conditions (heat, spot blotch or combined), X_p = mean Y_p of all genotypes under optimal planting conditions.

Separate values of SFSI were calculated for heat stress, spot blotch and combined stress for all the genotypes included. The genotypes were rated as highly tolerant (SFSI $\leq$ 0.50), moderately tolerant (0.50<SFSI$\leq$1.00) or susceptible (SFSI>1.00) to the particular stress (Fischer and Maurer, 1978; Khanna-Chopra and Viswanathan, 1999).

Cumulative growing degree-days (CGDD) were calculated for each year and sowing date, using 0°C as the base temperature from seedling germination to physiological maturity, as previously reported by Cao and Moss (1989). All statistical analysis was done using the SAS software (SAS, 2003). Normality was tested for all the response variables. Homogeneity of error variance between the two years was tested with the F test, as outlined by Gomez and Gomez (1984). Combined univariate and multivariate analysis of variance was done using the PROC ANOVA program of SAS (2003).

Results and discussion

Foliar blight severity was very high during the study period, as shown by the AUDPC value of the susceptible genotype BL 1473 (Fig. 1). Spot blotch was observed as early as the third week of February, after the wheat reached the heading stage. The disease symptoms were uniformly visible on all plants. Towards maturity, the disease severity reached 100% on the susceptible parent, but was below 25% on the resistant genotypes. Isolates from representative diseased leaf samples showed masses of *C. sativus* conidia on the lesions. No disease other than spot blotch was evident during the study period.

In the late-planted plots, heat stress was prominent in both years, as the average daily temperature was greater than 25°C for 23 days in 2006 and 25 days in 2007, in contrast with 3 days in 2006 and 4 days in 2007 for normal planting. Similarly, there were 28 days with a maximum temperature greater than 30°C in 2006 and 30 days in 2007 under late planting conditions. All days with a mean temperature higher than 25°C or a maximum temperature greater than 30°C from anthesis to maturity caused terminal heat stress. The value of CGDD was higher in late planting (2992 in 2006 and 3012 in 2007) compared to normal planting (2417 in 2006 and 2412 in 2007).

The genotypes showed variation in the level of disease severity (Fig. 1). Late planting generally increased the disease severity in all genotypes, although the magnitude differed with the genotype. The genotype NL971 was found to have a fairly high level of resistance in both years, whereas Gautam was moderately resistant, as indicated by the low disease progress values (AUDPC and AUDPC/day) in both years (Fig. 1). Also, both genotypes had low to moderate disease severity under late planting conditions.

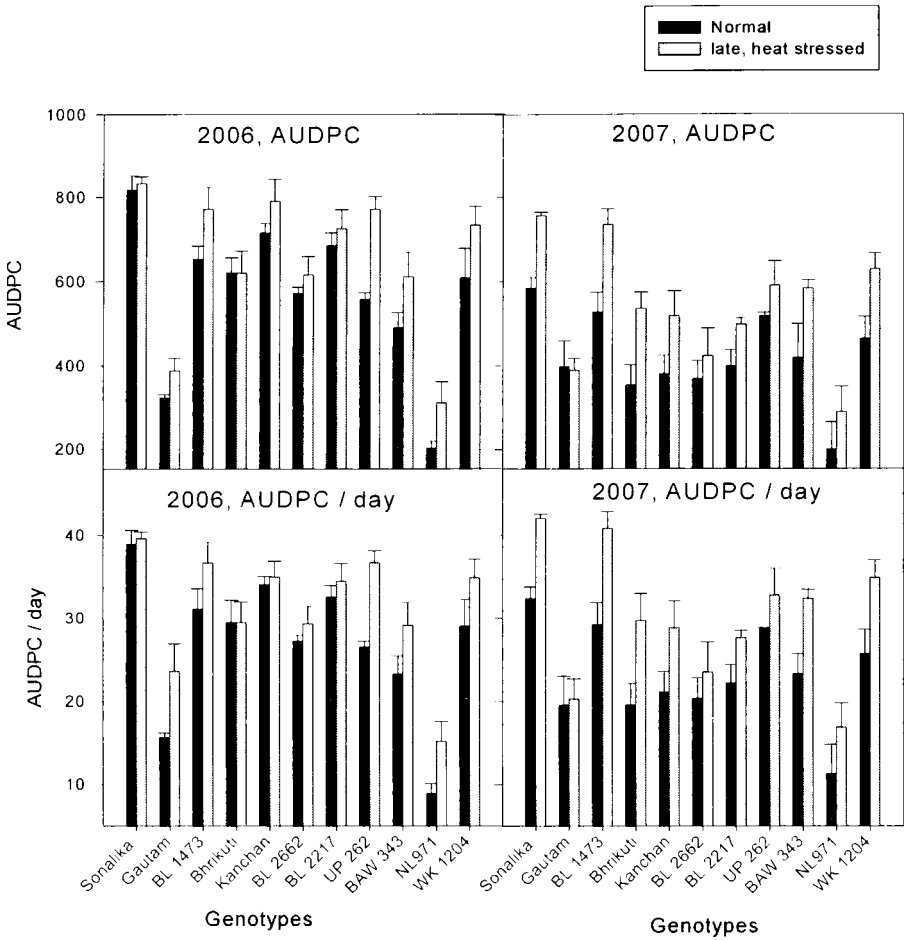

Fig. 1. Disease progress in genotypes, measured as area under the disease progress curve (AUDPC) and AUDPC/day. Error bars represent standard error of means

The effect of fungicide spray and date of planting had a significant effect on grain yield (GRY), thousand-kernel weight (TKW), biomass yield (BMY), days to heading (DH) and days to maturity (DM), but not on plant height (PH), number of grains per spike (GR/SP) or number of spikelets per spike (SPL/SP) (Table 1). Grain yield and thousand-kernel weight were the most affected components (Table 2). The obvious reason for this was that both disease and heat stress affect the grain-filling process, as they progressively increase after anthesis. The results are consistent with previous studies on terminal heat stress, where reduced yields were attributed mostly to lower kernel weight (Tashiro and Wardlaw, 1989). Kernel number was unaffected, in contrast to observations made in previous controlled environment studies (Gibson and Paulsen, 1999; Khanna-Chopra and Viswanathan, 1999). This difference could be attributed to the growth stage at which heat stress was present (Shpiler and Blum, 1986).

Table 1

Mean sum of squares for yield and yield components as affected by spot blotch and heat stress evaluated under field conditions at Rampur, Chitwan, Nepal during 2006–2007

Source	DF	AUDPC	AUDPC/day	GRY	TGW	BMY
Year (Yr)	1	8151	130.4	4069	533	957831
Error (a)	***6***	***18300***	***45.7***	***979961***	***177***	***7925341***
Fungicide Spray (spr)	1	–	–	2426708*	892**	18774090**
Yr × Spr	1	–	–	86983	120	28243
Error (b)	*6*	–	–	*380065*	*216*	*2344807*
Date	1	469823**	1462.4**	228083516**	15869*	1075412814**
Yr × Date	1	525691**	1610.3**	19009	782	9543740
Error (c)	***6***	***6856***	***16.4***	***731652***	***207***	***3717207***
Spr × Date	1	–	–	422837	1075*	2750280
Yr × Spr × Date	1	–	–	351292	1077*	1157789
Error (d)	***6***	***11954***	***32.3***	***167455***	***120***	***614260***
Genotype (Gen)	10	227451**	581.1**	1286353**	835**	10021841**
Gen × Date	10	18931**	49.3**	1011832**	124	5623393**
Spr × Gen	10	–	–	341862**	171	908731
Gen × Date	10	8452**	21.7**	157214	168	1153489
Yr × Gen	10	76622**	181.9**	522633**	195	3501046**
Yr × Gen × Date	10	11655**	30.0**	222003*	157	1453045
Yr × Spr × Gen	10	8319**	20.3**	76802	190	517437
Yr × Spr × Gen × Date	10	2008	4.7	171640	151	543396
Error (e)		***3015***	***7.7***	***117621***	***160***	***798464***

Source	DF	PH	DH	DM	GR/SP	SPL/SP	Multivariate†
Year (Yr)	1	1531*	834.6**	103	90793	112	0.0001*
Error (a)	***6***	199	8.3	4.4	3360	1.6	–
Fungicide Spray (spr)	1	10	2.6	31.3*	2828*	2	0.130**
Yr × Spr	1	12	7.7	29*	1685	0	0.120**
Error (b)	*6*	49	2.9	2.6	279	2.1	–
Date	1	23438**	7309.1**	29036.4**	1390	2.2	0.006**
Yr × Date	1	68	423.3	2601.8**	4364	11.9*	0.007**
Error (c)	***6***	79	17	12.6	1190	1.2	–
Spr × Date	1	40	3.3	22.5**	10668	0.1	0.203**
Yr × Spr × Date	1	118	1.1	29**	7699	0.5	0.198**
Error (d)	***6***	35*	1.4	1.7	4473	3.8	–
Genotype (Gen)	10	422**	116.3**	66.2**	20258**	31.5**	0.001**
Gen × Date	10	126**	23.3**	15.4**	2214*	2.1	0.006**
Spr × Gen	10	14	1.6	1.9	1619	1.6	0.146**
Gen × Date	10	17	3.8*	6.3**	611	1.4	0.134**
Yr × Gen	10	119**	72.4**	67.4**	17345**	10.5**	0.001**
Yr × Gen × Date	10	115**	15.9**	17.3**	1706	1	0.014**
Yr × Spr × Gen	10	3	1.3	3	1351	1.7	0.171**
Yr × Spr × Gen × Date	10	4	2.8	5.2	617	2.1	0.143**
Error (e)		14	1.7	1.4	1037	1.2	–

†Multivariate test was based on Wilks' likelihood ratio test ; * and ** significant at $p<0.05$ and $p<0.01$, respectively; Abbreviations: DF – Degrees of freedom, AUDPC – Area under disease progress curve, AUDPC/day – Area under disease progress curve/day, GRY – Grain yield, TKW – Thousand-kernel weight, BMY – Biomass yield, PH – Plant height, DH – Days to heading, DM – Days to maturity, GR/SP – Number of grains per spikelet, SPL/SP – Number of spikelets per spike

Table 2
Susceptibility index due to higher heat stress due to late planting, spot blotch and both, calculated for the grain yield and thousand-kernel weight of genotypes evaluated under field conditions at Rampur, Chitwan, Nepal

Genotypes	GRY† kg ha^{-1}	Susceptibility index for grain yield			TKW+ (g)	Susceptibility index for TKW		
		Heat	Spot blotch	Combined‡		Heat	Spot blotch	Combined‡
				Year 2006				
Sonalika	2901	1.05	1.24	1.27	46.8	1.35	1.54	1.39
Gautam	3370	0.32	0.74	0.38	48.6	0.60	0.46	0.46
BL 1473	3859	0.30	0.54	0.35	46.7	0.42	0.52	0.47
Bhrikuti	3331	0.96	1.12	1.03	46.8	1.33	1.44	1.22
Kanchan	3713	1.32	1.30	1.29	44.2	1.01	0.89	1.13
BL 2662	3724	1.52	1.38	1.32	48.4	1.27	1.10	1.07
BL 2217	3425	1.12	1.14	1.20	47.3	1.44	1.42	1.13
UP 262	3433	1.35	1.06	1.33	47.4	0.95	1.10	1.22
BAW 343	3476	1.43	1.35	1.18	52.2	1.10	1.17	0.80
NL971	3944	0.34	0.28	0.34	46.7	0.61	0.21	0.48
WK 1204	3141	1.44	0.99	1.51	45.1	0.91	1.13	1.69
D value		*0.45*	*0.23*	*0.52*		*0.21*	*0.16*	*0.39*
				Year 2007				
Sonalika	3457	0.94	1.41	1.15	42.1	0.84	1.68	1.06
Gautam	4122	0.57	0.55	0.51	46.6	0.57	0.52	0.57
BL 1473	4188	1.03	0.39	1.07	49.5	0.65	0.65	0.76
Bhrikuti	3853	0.96	1.34	0.92	48.1	1.34	1.36	1.34
Kanchan	3571	1.01	1.06	1.12	46.2	1.09	1.06	0.91
BL 2662	3766	1.45	1.27	1.22	43.5	1.09	1.00	0.70
BL 2217	3937	1.27	1.04	1.17	49.8	1.48	1.34	1.36
UP 262	3797	1.09	1.21	1.20	47.4	1.21	1.21	1.21
BAW 343	3270	0.87	1.52	1.05	46.2	1.01	0.96	0.96
NL971	4165	0.45	0.33	0.53	44.7	0.45	0.26	0.81
WK 1204	4188	1.38	1.14	1.14	46.5	1.21	0.98	1.26
D value		*0.20*	*0.43*	*0.44*		*0.27*	*0.17*	*0.31*

† Grain yield under optimum planting conditions (normal sowing date, disease protection); ‡ combined stress plots, with higher heat stress due to late planting and spot blotch due to no fungicide protection; +TKW under optimum planting conditions (normal sowing date, disease protection); D value = stress intensity

Heat stress during GS3 (anthesis to maturity) mainly affects assimilate availability, the translocation of photosynthates to the grain, and starch synthesis and deposition in the developing grain. Increased mean temperature in the range of 12–26°C has been found to be associated with reduced grain filling (Wiegand and Cuellar, 1981). TKW has been reported to be most affected by high spot blotch severity (Sharma and Duveiller, 2004; Rosyara et al., 2007), heat stress (Sharma et al., 2008) and spot blotch combined with heat stress (Sharma and Duveiller, 2004). Spot blotch development and increased temperature affect wheat simultaneously in South Asian environments, producing similar consequences. Spot blotch has also been associated with reduced grain yield due

to a reduction in TKW, without affecting components that are determined preanthesis (Rosyara et al., 2007). When spot blotch and heat stress are combined, there is an increase in the severity of spot blotch (Sharma and Duveiller, 2004).

The resistant genotype NL971 and the moderately resistant genotype Gautam had lower grain yield reduction in both years under all three types of stress conditions compared to the other genotypes (Fig. 2). When spot blotch and heat stress were combined the yield reduction was substantial even for these genotypes. Interestingly, genotype BL 1473 had lower yield reduction (Fig. 2) despite high disease severity (Fig. 1). The response was consistent in both years, showing that it possessed some tolerance. It seems that yield losses due to disease are more severe under late planting conditions. Both fungicidal protection and normal date of planting helped to reduce the extent of yield loss. The results are consistent with the severe yield reduction reported due to spot blotch (Rosyara et al., 2007), heat stress (Sharma et al., 2008; Randall and Moss, 1990) or combined stresses (Sharma and Duveiller, 2004).

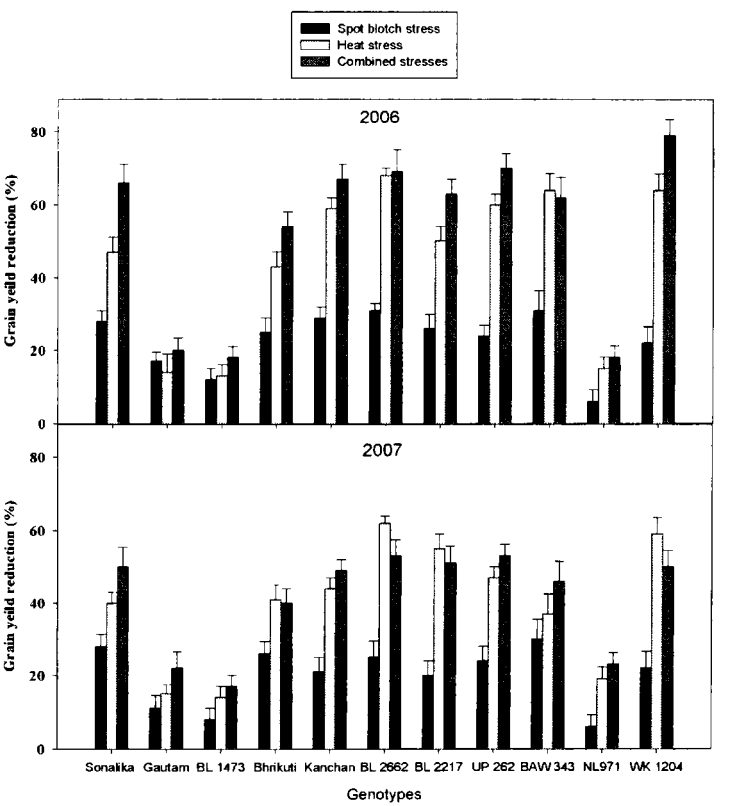

Fig. 2. Reduction in grain yield due to heat and spot blotch stresses in different genotypes grown in Rampur, Chitwan, Nepal during 2006 and 2007

Variation among the genotypes was observed for the stress factor susceptibility index (SFSI) based on grain yield and TKW. Based on SFSI, NL971, Gautam and BL 1473 were found to be highly tolerant (SFSI $\leq$ 0.50) to moderately tolerant (0.50<SFSI$\leq$1.00) for spot blotch, heat stress or combined stresses in both years (Table 2). The other genotypes were found to be sensitive to this type of stress. The genotypes responded in a similar way to both types of stress, individually or combined. Based on disease severity, NL971 is a resistant genotype (Fig. 1), which exhibits the stay green trait, which might help it to tolerate terminal heat stress. Gautam has a moderate level of resistance, whereas BL 1473 is highly susceptible to spot blotch (Fig. 1).

Diversity for heat stress tolerance is well established (Al-Khatib and Paulsen, 1990; Reynolds, 1994; Lillemo et al., 2005). Spot blotch tolerance is not well known, and researchers have paid less attention to tolerance than to resistance. Tolerance was first suspected in experiments where the grain yield or yield losses due to the disease were not well correlated with spot blotch severity (Sharma et al., 2007b). Consistently with the results of this study, one very susceptible genotype, BL 1473, was reported to have tolerance to spot blotch in different studies (Sharma et al., 2007b; Rosyara et al., 2007; Duveiller et al., 2005). BL 1473 has been shown to respond to the artificial removal of photosynthetically active leaves by demonstrating some type of mechanism to compensate for the reduced supply of photosynthates (Rosyara et al., 2005). In the case of both disease pressure and artificial defoliation, the genotype responded with a less pronounced reduction in thousand-kernel weight and grain yield.

Responses to spot blotch and heat stress tolerance were similar. The genotypes responded in a similar way to both the stresses, either individually or combined, suggesting the existence of similar physiological mechanisms to promote tolerance of both types of stress. Along with spot blotch resistance, various levels of tolerance to both stresses also exist. The results are applicable for the development of genotypes for warm, spot blotch-stressed environments.

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ANALYSIS OF HEAT STRESS TOLERANCE IN WINTER WHEAT

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As a consequence of climate change, the incidence of extreme weather events has increased in Hungary, as elsewhere. Extremely high temperatures are the factor causing the greatest problems for agriculture and crop production. The aim was to determine the heat tolerance of two wheat varieties (Plainsman V. and Mv Magma) by measuring physiological and yield parameters under high temperature conditions (35/20°C day/night) in the phytotron. Heat stress had a substantial influence on the chlorophyll content, antioxidant enzyme activity and yield parameters of the two winter wheat varieties. Heat stress during grain filling led to a significant reduction in the yield, biomass, grain number, harvest index and thousand-kernel weight. Significant differences could be detected between the two varieties, confirming the greater heat sensitivity of Plainsman V. and the better heat tolerance of Mv Magma. The importance of the antioxidant enzyme system was demonstrated in defence against heat stress. The activity of the enzymes glutathione-S-transferase (GSH-S-Tr), ascorbate peroxidase (APx) and catalase (CAT) was enhanced in Plainsman V., and that of GSH-S-Tr and CAT in Mv Magma. The tolerance of the wheat varieties appeared to be correlated with the antioxidant level, though changes in activity were observed for different antioxidant enzymes in the two genotypes tested.

Key words: heat stress, yield, chlorophyll content, antioxidant enzyme activity

Introduction

Plants are exposed to numerous stress effects that limit their development and yield. According to Larcher (1987), stress first leads to the destabilisation of plant functions, followed by normalisation and an increase in resistance. However, if the threshold of tolerance is crossed, it may result in long-term damage or even the death of the plant. Stress thus involves both destructive and constructive elements.

Temperatures exceeding the limit of adaptation cause heat stress, which has a substantial influence on the metabolism, plant viability and possibly on the ability of the plants to resist various pathogens.

High temperature damages processes responsible for light harvesting and the conversion of light energy and increases the rate of photorespiration. The light-harvesting chlorophyll-protein complex may be irreversibly detached from the nucleus of the reaction centre and the water-decomposing system responsible for oxygen production may also suffer. Damage to the chloroplast membranes may also occur. All these factors have a negative effect on photosynthesis and respiration processes (Bernacchi et al., 2002; Balla et al., 2008).

As the result of changes induced by temperature stress, toxic reactive oxygen species may also accumulate in the plant cells. These also occur in small quantities under normal conditions, as a consequence of the functioning of the antioxidant system, without causing damage to the cells. However, when their quantity increases in response to various stress factors they may damage cell components and cause severe disturbances (Janda et al., 2003; Kocsy et al., 2002). Stress tolerance is thus based on the activation of the antioxidant systems, which are capable of neutralising the reactive oxygen species (ROS) formed in response to stress, and thus preventing them from damaging or killing the cells.

Almeselmani et al. (2006; 2009) gave proof of the increase in enzyme activity as a result of heat stress, reporting enhanced superoxide dismutase (SOD), ascorbate peroxidase (APx) and catalase (CAT) activity in wheat in the vegetative phase, at flowering and 15 days after flowering in the case of late or extremely late sowing. At the same time, a reduction in the activity of the glutathione reductase (GR) and peroxidase (POX) enzymes was observed, compared with the normal sowing date (Almeselmani et al., 2006).

Under other stress conditions, e.g. drought, it was also observed that the activity of some antioxidants increased, while that of others decreased (D'Souza et al., 2009; Esfandiari et al., 2008). The activity of ascorbate (ASC) and glutathione (GSH) was found to increase and that of SOD and CAT to decrease in more severe treatments. Of these two antioxidant enzymes, the low activity of catalase could have been responsible for the appearance of higher quantities of H_2O_2, which then caused disturbances in the functioning of the H_2O_2-sensitive enzymes involved in the Calvin cycle (Yamazaki et al., 2003).

In response to stress, the relative water content of the leaf and the quantity of chlorophyll in the leaves decline rapidly, and the plants turn yellow and reach harvest maturity far earlier than the control plants (Jiang and Huang, 2001). If the plant is exposed to stress during ripening, aging processes accelerate, thus shortening the period available for grain development and leading to considerable yield losses.

The present work investigated the effect of the increasing occurrence of high temperatures on the antioxidant activity in winter wheat.

Materials and methods

The effect of heat stress during the grain-filling phenophase of cereals was investigated under controlled conditions in the Martonvásár phytotron. On the basis of preliminary studies, the winter wheat varieties Plainsman V. (USA) and Mv Magma (H) were chosen for the tests.

For each variety, germinated seeds were planted four to a pot in a 3:2:1 mixture of soil, Vegasca and sand, and raised in the phytotron. Heat treatment was commenced 12 days after heading and continued for 15 days. Day/night temperatures of 24/20°C were programmed in the control treatment (C) and 35/20°C for 8 h a day in the stressed treatment (H) (Tischner et al., 1997).

The chlorophyll content was determined using a Minolta SPAD-502 Chlorophyll Meter, which calculates SPAD values proportional to the chlorophyll content on the basis of leaf transmittance. The chlorophyll content was measured in four replications per pot and the results were evaluated using two-factor analysis of variance.

The effect of heat stress was also examined using the agronomic parameters of the two wheat varieties. Measurements were made of the total biomass, total grain number and total grain mass per plant, and the harvest index and thousand-kernel weight were calculated.

Leaf samples of both varieties were collected from the above treatments in five replications on the 1st and 7th day of the stress treatment for the analysis of antioxidant enzyme activity. The samples were stored at –85°C prior to measurement. The enzyme activity was recorded photometrically (Shimadzu UV-VIS 160A) at room temperature. Five antioxidant enzymes were investigated: glutathione reductase (GR), glutathione-S-transferase (GSH-S-Tr), ascorbate peroxidase (APx), catalase (CAT) and guaiacol peroxidase (GPx).

Measurement of enzyme activity

In the case of CAT, 0.5 g leaf tissue was homogenised in 2.5 ml chilled Tris buffer (pH 7.4, 0.5 M). The reaction mixture contained 0.5 mM Tris buffer (pH 7.4), 10 mM H_2O_2 and 50 µl plant sample, in a total volume of 3 ml. The CAT activity of the plant extract was measured spectrophotometrically by monitoring the decrease in absorbance at 240 nm.

The reaction mixture for APx activity measurements contained 0.25 mM ascorbic acid and 0.5 mM H_2O_2 in Tris buffer (pH 7.8), and the activity was recorded at 290 nm (decreasing absorbance).

The GR activity was determined at 412 nm using the method of Smith et al. (1988). The reaction mixture consisted of 75 mM phosphate buffer (pH 7.5), 0.15 mM DTPA (diethylenetriamine-pentaacetic acid), 0.75 mM DTNB [5,5'-dithiobis(2-nitrobenzoic acid)], 0.1 mM NADPH, 0.5 mM oxidised glutathione (GSSG) and 50 µl plant extract in a total volume of 1 ml.

GPx activity was determined as described by Ádám et al. (1995). Changes in absorbance due to the oxidation of guaiacol were recorded spectrophotometrically at 470 nm. The reaction mixture contained 1 ml 1 mM guaiacol and 50 µl plant sample in 0.1 M Na-acetate buffer (pH 5.5), and the reaction was initiated by the addition of 300 µl 1.3 mM H_2O_2 solution.

The activity of GSH-S-Tr was recorded as the change in absorbance at 340 nm in a reaction mixture containing 72.7 mM Na-phosphate buffer (pH 6.5), 3.6 mM reduced glutathione (GSH), 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 100 µl plant extract.

Results

Changes in chlorophyll content and yield in response to heat stress

The responses of the varieties gave a good illustration of the effect of the extreme heat stress applied. The differences in the heat tolerance of the two varieties were most apparent in the changes in chlorophyll content (Fig. 1). There was a clearly visible decline in the chlorophyll content on the 2nd day in Plainsman V., while that of Mv Magma exhibited no change compared with the control.

The stress treatment also caused substantial changes in the yield parameters (Fig. 2), with significant differences between the two varieties. The better heat tolerance of Mv Magma was clear from the less severe reductions in biomass, grain yield, thousand-kernel weight, harvest index and grain number, while Plainsman V. was less able to tolerate a temperature of 35°C, particularly in terms of biomass, grain yield and thousand-kernel weight.

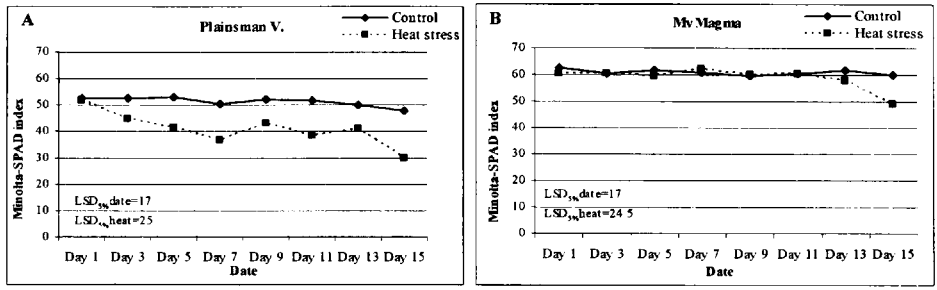

Fig. 1. Changes in the chlorophyll content of Plainsman V. (A) and Mv Magma (B) in response to heat stress. $LSD_{5\%}$ represents the minimal difference between the genotypes which is statistically significant at the $P \leq 0.05$ level.

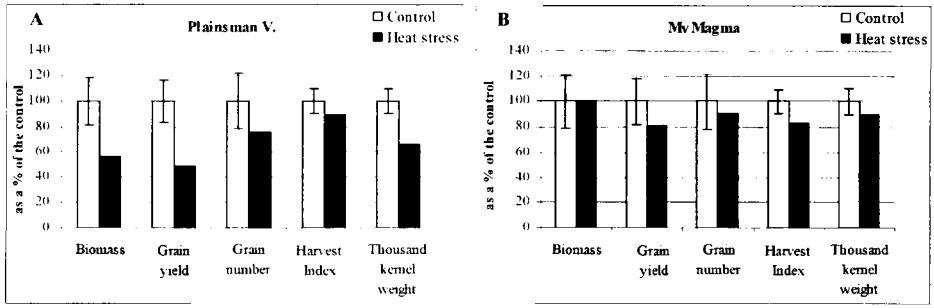

Fig. 2. Changes in the yield parameters of Plainsman V. (A) and Mv Magma (B) in response to heat stress

The results confirmed that heat stress during grain filling causes a significant reduction in almost all the yield parameters. The significant differences in abiotic stress tolerance between the genotypes suggest that resistance to these factors could be improved by breeding.

Changes in antioxidant enzyme activity in response to heat stress

The enzyme activity of the two winter wheat varieties was analysed in adult plants during the grain-filling stage. Leaf samples were taken on the 1st and 7th days of the stress treatment from control and heat-stressed plants. Significant differences in antioxidant enzyme activity could be detected between the varieties (Fig. 3).

In response to heat stress there was no significant change in the GR activity of Plainsman V., while in Mv Magma there was a significant decrease on both the 1st and 7th days of treatment. This could also have been due to the heat stress sensitivity of GR, leading to enzyme degradation. It was also observed that the activity of this enzyme declined as the plants aged.

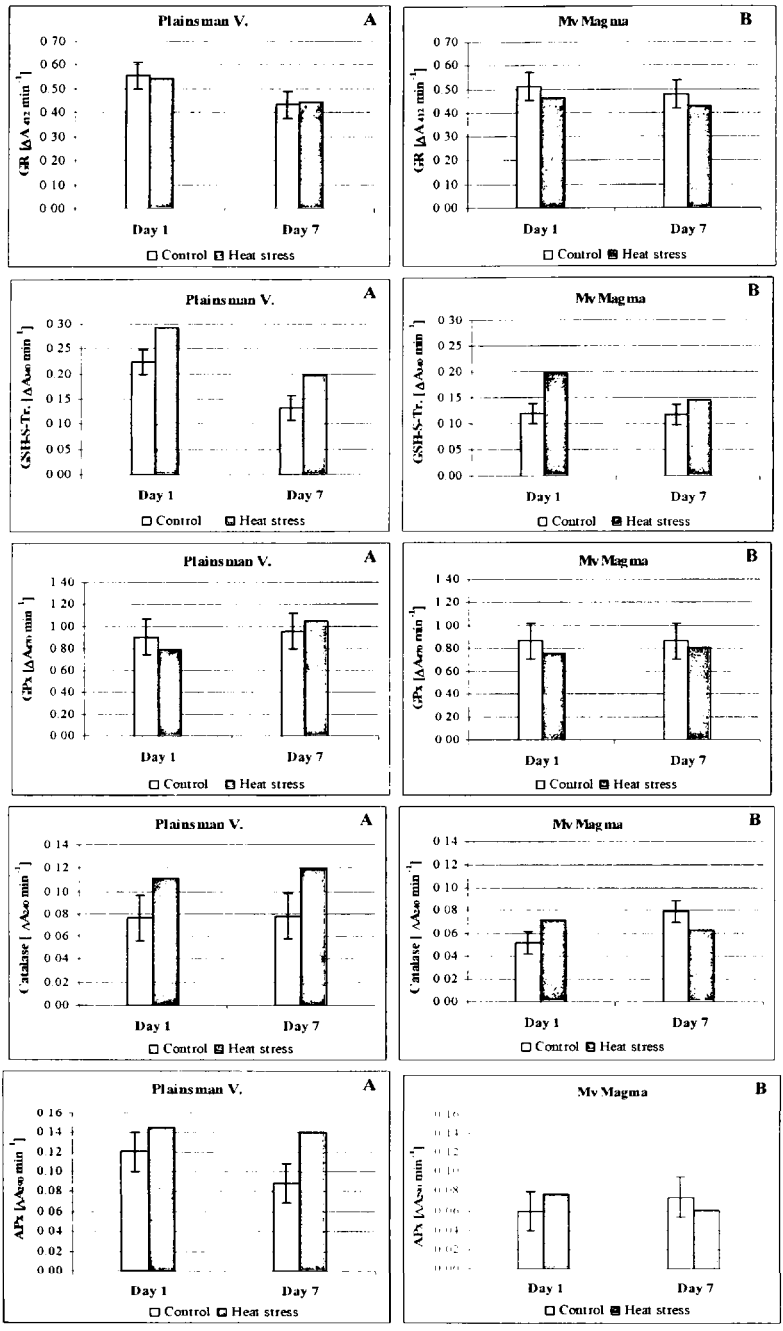

Fig. 3. Activities of glutathione reductase (GR), glutathione-S transferase (GSH-S-Tr.), guaiacol peroxidase (GPx), catalase and ascorbate peroxidase (APx) expressed as changes in absorbance in enzyme extracts prepared from control and heat-stressed samples of Plainsman V. (A) and Mv Magma (B) during the grain-filling stage. Bar: differences significant at the $P \leq 0.05$ level.

In response to heat stress a significant increase was observed in the activity of the glutathione-S-transferase (GSH-S-Tr) enzyme in both varieties. This could have contributed to plant resistance by neutralising the damaging effects of the reactive oxygen species and free radicals formed in the course of stress. For this enzyme, too, the activity on the 7th day of stress treatment was considerably lower than on the 1st day, possibly due to the aging of the plants.

In the case of the guaiacol peroxidase (GPx) enzyme no significant increase or decrease in enzyme activity was detected for either variety after high temperature treatment.

The greatest increase in enzyme activity in response to heat stress was recorded in the case of catalase. At both sampling dates there was a significant rise in the activity of this enzyme in Plainsman V., while in Mv Magma an initial rise was later followed by a significant reduction in activity.

In Plainsman V. the rise in the activity of the ascorbate peroxidase (APx) enzyme in response to high temperature was similar to that observed for catalase at both sampling dates. This could have helped to compensate for the greater heat sensitivity of the variety. In the case of Mv Magma no significant changes were observed for this enzyme.

Discussion

The phytotron experiments on physiological traits and yield proved that the temperature plays a major role in the yield levels achieved by wheat. The difference in heat sensitivity between the varieties tested (Plainsman V. and Mv Magma) was clearly illustrated by both the chlorophyll content and the yield parameters. On the basis of chlorophyll content Mv Magma had good tolerance of high temperature throughout the experiment, while Plainsman V. exhibited a considerable decline even at the beginning of treatment. This was in agreement with the findings of Jiang and Huang (2001) and Bencze et al. (2005), who reported that the leaves rapidly turned yellow at high temperature, with a gradual decrease in the chlorophyll content, suggesting a drastic reduction in photosynthetic activity.

Heat stress also had a substantial effect on the yield parameters of both varieties. Significant differences could be detected between the two varieties, confirming the greater sensitivity of Plainsman V. to high temperature (>35°C) compared to Mv Magma.

The plants attempted to improve their resistance to heat stress through the greater activity of the antioxidant enzymes, but some enzymes gave little or no response to heat shock, suggesting that they were degraded by high temperatures. Other enzymes, however, such as catalase, ascorbate peroxidase and glutathione-S-transferase, exhibited a significant increase in activity following treatment.

Other authors also reported genotypic differences in the activation of the ascorbate peroxidase enzyme, a key compound for the metabolisation of H_2O_2 (Balla et al., 2007; Dash and Mohanty, 2002; Janda et al., 2003; Veisz et al., 2004). Northern blot analysis was used to prove the rise in APx gene expression in response to heat stress, both in the vegetative stage and at flowering (Almeselmani et al., 2009). Catalase, which utilises H_2O_2, was also stimulated in seedlings by non-lethal temperatures of 30–35°C, but stress treatment at 40°C led to the inactivation of the enzyme (Dash and Mohanty, 2002). This was confirmed by the present results as, although the plants were treated in a different phenophase, certain enzymes were inactivated. Sabeva and Nedeva (2008) also reported that catalase was the antioxidant enzyme most severely affected by chronic stress.

The results revealed more intensive enzyme activity in Plainsman V. than in Mv Magma, which appeared to be correlated with the greater heat sensitivity also demonstrated in this work. As the plants were more sensitive to 35°C heat stress, they tried to increase their chances of survival by producing more antioxidants. However, the increased antioxidant enzyme activity was only sufficient to compensate for the damaging effect of heat stress, being unable to increase the yield. In the case of Mv Magma, the rise in antioxidant enzyme activity was less pronounced. This slighter, but significant, change was sufficient to protect the more heat-tolerant plants against the damaging effects of heat stress. At the same time, the reduction in enzyme activity may have had an influence on the development of defence mechanisms in the plants.

It is clear from the findings of Sairam et al. (2000) that enhanced antioxidant enzyme activity in response to stress does not necessarily indicate that a genotype is tolerant of the given stress. In addition, although the tolerance of various wheat varieties may be correlated with the activity of antioxidant enzymes capable of counteracting or mitigating oxidative stress, different enzymes may be activated in different genotypes. This is a further indication of the complexity of the processes that have developed in plants over time to achieve adaptability to various stress factors, while the investigation of these processes is complicated by the genetic variability of the varieties.

Acknowledgements

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ENZYMATIC ANTIOXIDANT DEFENCE MECHANISMS OF MAIZE AND SORGHUM AFTER EXPOSURE TO AND RECOVERY FROM PRE- AND POST-FLOWERING DEHYDRATION

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Drought stress is often the most limiting factor for maize and sorghum production in the semi-arid areas. This study evaluates the enzymatic antioxidant protection mechanism response of maize (cv Melkassa-2) and sorghum (cv. Macia) after exposure to and recovery from pre- and post-flowering dehydration.

The response of enzymatic antioxidant protection systems revealed that in both test crops dehydration during both the pre- and post-flowering stages resulted in increased activities of enzymatic antioxidant protection mechanisms (SOD, GR, CAT and APX). There were, however, differences between the species in the type and extent of enhanced developmentally-induced and dehydration-induced antioxidant activities. Differences were also noticed in the relative water contents at which changes in enzymatic antioxidant activities occurred. Under dehydration conditions, sorghum was generally found to have relatively higher enzymatic antioxidant activities, providing it better protection against oxidative stress by minimizing the level of lipid peroxidation.

Lipid peroxidation, measured as MDA content, was increased in both species during pre- and post-flowering dehydration, but the increase was greater in maize than in sorghum during both developmental stages. Sorghum appeared to be able to reduce MDA on rehydration, but maize contained only 85% less MDA after rehydration as compared to the control following pre-flowering rehydration. During post-flowering rehydration, neither species was able to decrease the MDA content to the control level.

The results indicated that tolerance to drought in sorghum is well associated with the consistent enhanced capacity of the enzymatic antioxidant system under both pre- and post-flowering dehydration conditions, and that the sensitivity of maize to drought is linearly correlated to the decreased capacity of the antioxidant system. It may be concluded that, since differences were observed between the species in the response of enzymatic antioxidants to pre- and post-flowering dehydration/rehydration, with sorghum exhibiting comparatively higher overall activities of enzymatic antioxidants and a lower level of MDA than maize during both pre- and post-flowering dehydration, selection based on these criteria may help in the development of genotypes tolerant to dehydration.

Key words: dehydration, enzymatic antioxidants, malondialdehyde, reactive oxygen species, rehydration

Introduction

Plant growth and productivity is adversely affected by various environmental factors and water deficit stress is considered to be one of the most important causes of decreased grain yield in cultivated crops. Genetic improvement for drought tolerance is, therefore, of particular importance to agricultural plants. Drought stress may lead to stomatal closure, which reduces CO_2 availability in the leaves and inhibits carbon fixation, exposing chloroplasts to excessive excitation energy, which in turn could increase the generation of reactive oxygen species (ROS) and induce oxidative stress (Reddy et al., 2004). The ROS which are generated in response to drought stress include superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($O_2^{\cdot}$) (Mittler, 2002). The excess production of ROS during drought stress results from impaired electron transport processes in the chloroplasts and mitochondria (Smirnoff, 1993). The superoxide radical is generated at the membrane level in most plant cell organelles and hydrogen peroxide is the product of superoxide dismutase (SOD) and of several oxidases of the peroxisomes (Thompson et al., 1987). These ROS are highly reactive and affect lipid peroxidation, protein denaturation and DNA mutation (Pastori and Foyer, 2002; Apel and Hirt, 2004).

To minimize the damaging effects of ROS, plants have evolved various complex antioxidant systems which are composed of enzymatic and non-enzymatic defensive systems that can reduce oxidative stress by scavenging reactive oxygen species (Alscher et al., 2002; Shigeoka et al., 2002; Apel and Hirt, 2004). The enzymatic antioxidant defence mechanisms are represented by superoxide dismutase, which catalyses the dismutation of superoxide radicals to H_2O_2 and O_2 (Smirnoff, 1993), while ascorbate peroxidase (APX) and catalase break down H_2O_2 to water (Sairam et al., 2002; Mittova et al., 2002). In chloroplasts, H_2O_2 is also eliminated by the action of the ascorbate glutathione cycle, where glutathione reductase and ascorbate peroxidase are the key enzymes (Foyer et al., 1994).

The enhancement of antioxidant defence mechanisms is considered to be an adaptive mechanism of plants to drought stress. The available studies suggest a correlation between stress tolerance and antioxidant defence capacity (Sgherri and Navari-Izzo, 2000; Acar et al., 2001; Reddy et al., 2004). The damaging effects of an externally imposed biotic or abiotic stress can be partly attributed to the over-riding of existing resistance mechanisms. Only when those mechanisms are overwhelmed would injury occur (Smirnoff, 1993; Zhang and Kirkham, 1996). This indicates that the strengthening of the defence mechanisms, through the enhanced functions of their components, such as superoxide dismutase, ascorbate peroxidase, glutathione and catalase, may reduce or prevent oxidative damage and improve the drought resistance of plants. It was noted that antioxidant activity responds differently to water deficit in different species

(Smirnoff, 1993). Previous studies have indicated that under drought stress, an increase in antioxidant activities was reported in dehydration-induced abiotic stresses in tomato (Mittova et al., 2002), maize (Azevedo Neto et al., 2006), sorghum and sunflower (Zhang and Kirkham, 1996), implying their role in the acquisition of drought tolerance. Although several studies strongly suggest that the accumulation of antioxidants plays a drought tolerance role, there is very little information on the correlation of their expression and the level of drought tolerance in different crop types. A better understanding of the physiological and metabolic changes and tissue responses to water deficit may promote the selection of drought-tolerant crop species and the elucidation of the underlying control mechanisms.

This study was therefore aimed at investigating the accumulation of antioxidant enzymes and at comparing the expression of these traits and the level of drought resistance in maize and sorghum after exposure to and recovery from pre- and post-flowering dehydration.

Materials and methods

Plant material, growth conditions, treatments and experimental design

The seed materials for this study, maize (*Zea mays* L.) cv. Melkassa-2 and sorghum (*Sorghum bicolor* (L.) Moench) cv. Macia were obtained from the Division of Maize Improvement for Moisture Stress Areas, Melkassa Agricultural Research Centre, Ethiopia and ICRISAT Centre, Bulawayo, Zimbabwe, respectively. Five seeds of each species were planted in pots (each 31 cm deep with an internal diameter of 18 cm) containing 10 kg of loam soil and allowed to grow under a constant environment of 12/12 h day/night, 28–32/17°C day/night temperature, 60–80% RH and 1200–1400 μmol $m^{-2}s^{-1}$ PPFD at the Department of Botany, University of Cape Town. Twenty days after emergence the pots were thinned to two seedlings of uniform stand per pot. The plants were watered frequently to avoid the development of moisture deficit. Starting from sixty (pre-flowering) and ninety days (post-flowering, grain-filling stage) after emergence, two watering treatments were applied: control (fully hydrated) or dehydrated treatments. Control plants were regularly watered to avoid the development of water stress, while dehydration was induced by withholding water for 20 days at each growth stage. Subsequently, plants dehydrated at each growth stage were rehydrated for 20 days and their recovery was studied.

The pots were factorially arranged in a randomized complete block design (RCBD) with four replications. Five different samples were taken during the dehydration period at each growth stage and during recovery. Each pot was given P and N at the rate of 0.80 g pot^{-1} (150 kg ha^{-1}) and 1.1 g pot^{-1} (200 kg ha^{-1}), respectively. Single superphosphate and lime ammonium nitrate were used as the source of P and N, respectively. The parameters detailed below were measured at regular intervals during the entire cycle (pre- and post-flowering dehydration). The same measurements were performed on control plants, which remained hydrated throughout.

For each antioxidant enzyme activity and for lipid peroxidation three sample extracts were made per plant.

Assays of antioxidant enzyme activities

Leaf samples of approximately 1 g fresh weight were taken for the determination of enzymatic antioxidant activities, immediately frozen in liquid nitrogen and stored at −80°C until analysis.

Frozen leaf samples weighing 0.5 g were ground into a fine powder using a chilled mortar and pestle under liquid N. The leaf samples were extracted by homogenizing the powder in 0.1 M phosphate buffer (pH 7.8) containing 2 mM DTT, 0.1 mM EDTA and 1.25 mM PEG 4000. Insoluble material was removed from the homogenate by centrifugation at 11,500 rpm for 15 minutes at 4°C and the supernatant was used for the following enzyme assays. The total protein content in the enzyme extracts was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Superoxide dismutase (SOD) (EC 1.15.1.1)

SOD activity was assayed by monitoring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Giannopolitis and Reis (1977). The 3 ml reaction mixture contained 2.20 ml of 0.1 M phosphate buffer (pH 7.8), 0.25 ml of 1.3 µM riboflavin, 0.25 ml of 13 mM methionine, 0.25 ml of 63 µM NBT and 50 µl enzyme extract. Riboflavin was added last. Blank assays without extracts (water in place of sample) were also prepared. The cuvettes containing the reaction mixtures and blanks were placed under high light intensity lamps. The reaction was initiated by switching on the light, which was switched off after 15 minutes. A non-irradiated reaction mixture that did not develop colour served as the control. The reaction mixture that lacked enzyme developed maximum colour as a result of the maximum reduction of NBT. One unit of SOD activity was defined as the amount of enzyme necessary to inhibit the reduction of NBT by 50% as monitored spectrophotometrically (Beckman DU 650, USA) at 560 nm.

Glutathione reductase (GR) (EC 1.6.4.2)

GR activity was determined by following the oxidation of NADPH at 340 nm for 5 minutes at 25°C in 400 µl of an assay mixture containing 200 µl of 0.1 M phosphate buffer (pH 7.8), 50 µl of 3 mM $MgCl_2$, 25 µl of 10 mM GSSG, 25 µl of 0.5 mM NADPH and 100 µl enzyme extract. A correction was made for the background absorbance at 340 nm without NADPH (Schaedle and Bassham, 1977). The activity of GR was calculated from the rate of oxidation of NADPH, using an extinction coefficient of 6.22 mM^{-1} cm^{-1}. One enzyme unit was defined as µmol mg^{-1} protein min^{-1}.

Catalase (CAT) (EC 1.11.1.6)

CAT activity was determined by following the consumption of H_2O_2 at 240 nm (spectrophotometer Beckman DU 650) for 5 minutes at 25°C. The 3 ml reaction mixture for the determination of catalase contained 2.55 ml of 50 mM phosphate buffer (pH 7.0), 250 µl of 37.5 mM H_2O_2 and 200 µl of enzyme extract. One unit of enzyme activity was defined as the amount necessary to decompose 1 µM of H_2O_2 min^{-1} at 25°C, calculated from the extinction coefficient for H_2O_2 at 240 nm (0.0436 μmol^{-1} cm^2).

Ascorbate peroxidase (APX) (EC 1.11.1.1)

Frozen leaf samples (0.5 g) were ground into a fine powder using a chilled mortar and pestle under liquid N_2 The fine powder was extracted by homogenization in 10 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone (PVP), with the addition of 1 mM ascorbic acid (ASC). The reaction mixture of 1 ml contained 500 µl of 50 mM potassium phosphate buffer (pH 7.0), 15 µl of 0.5 mM ASC, 15 µl of 0.1 mM H_2O_2, 200 µl of enzyme extract and 270 µl H_2O. The reaction was started by adding H_2O_2. The homogenate was centrifuged at 15,000 *g* for 20 min at 4°C. APX activity was determined by following the decrease at 290 nm, using an extinction coefficient of 2.8 mM^{-1} cm^{-1} for 1 min.

Determination of lipid peroxidation

The extent of lipid peroxidation in the leaf segments was determined in terms of the malondialdehyde (MDA) content (a product of lipid peroxidation) by the thiobarbituric acid reaction using the method of Dhindsa et al. (1981). Approximately 0.5 g leaf sample was

homogenized in 6 ml of 0.1% trichloroacetic acid. The homogenate was centrifuged at 10,000 *g* for 10 min, after which 4 ml of 20% trichloroacetic acid containing 0.5% thiobarbituric acid was added to 1 ml aliquots of the supernatant. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice bath. After centrifuging the tube again at 10,000 *g* for 10 min, the absorbance of the supernatant was read spectrophotometrically (Beckman DU 650, U.S.A) at 532 nm. The value for the non-specific absorption at 600 nm was subtracted from the 532 nm reading. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer, 1968).

For each enzymatic and non-enzymatic antioxidant and MDA assayed, triplicate extractions were performed for each species and treatment at each growth stage.

Statistical analysis

Statistical analyses were carried out using STATISTICA for windows Version 6.0, Statsoft, Inc., USA. The results presented are the means of three replicates. In all figures, means were calculated and the significance of differences between the means were determined by variance analysis and Duncan's multiple range test at the 5% level of significance. Standard errors are represented as vertical bars.

Results

The results indicated that SOD activity was markedly increased by dehydration treatment in both species (Fig. 1). In maize, during pre-flowering dehydration the SOD activity increased immediately after withholding water. The increase was gradual until the RWC (Relative Water Content) reached 56%, and thereafter rapid. During post-flowering dehydration, however, the activity was between 200% and 150% of the control until RWC reached 56%, after which there was a sharp increase followed by a sudden decrease (Fig. 1a). In sorghum plants exposed to both pre- and post-flowering dehydration, there was a continual increase in SOD activity throughout the duration of the dehydration cycle (Fig. 1b). In both species, the activity of SOD was maximal during post-flowering dehydration at RWCs of 47% and 41% in maize and sorghum, respectively.

When maize plants that had been dehydrated during the pre-flowering stage were rehydrated, the SOD activities immediately decreased to the control level, whereas plants dehydrated during the post-flowering stage recovered only 50%, which still represented a 1.5-fold reduction compared to the control. In sorghum plants undergoing pre-flowering dehydration, approximately 80% of the SOD activities recovered to the control level, and in those undergoing post-flowering dehydration it decreased to the control level on rehydration (Fig. 1c and d).

Dehydration also induced an increase in GR activity in both species (Fig. 2). As for SOD activity, there were differences between the species with changes in RWC during dehydration. In maize, GR activity during pre-flowering dehydration peaked (368% of the control) at RWCs of about 55%, and thereafter activity dropped to approximately 206% with an increase in the intensity of dehydration at 47% RWC (Fig. 2a). During post-flowering dehydration, there was no noticeable change in GR activity until the RWC reached about 55%, after which there was a marked increase to a maximum of 205% at approximately 45% RWC. In contrast to maize, the GR activity in sorghum was enhanced continually and peaked at 387% and 240% at 41% RWC during pre- and post-flowering dehydration, respectively.

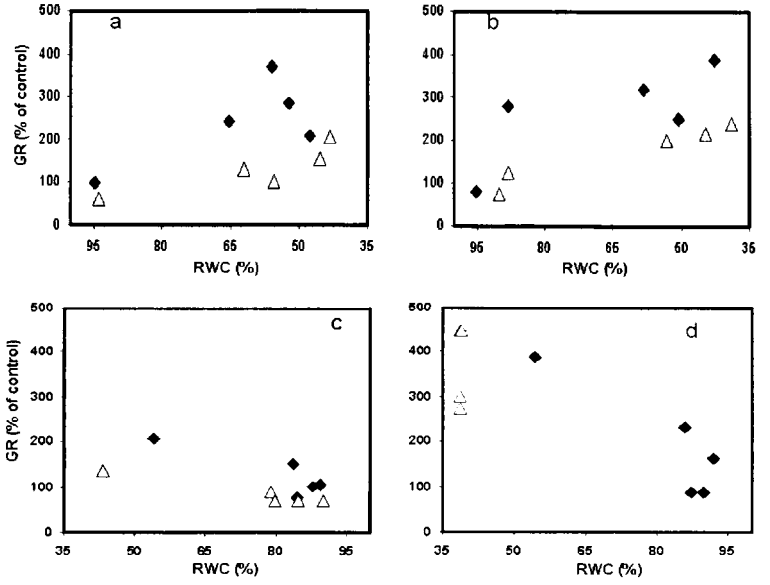

Fig. 1. Superoxide dismutase activities (% of control) in maize (a, c) and sorghum (b, d) during pre- and post-flowering dehydration (a, b) and rehydration (c, d) as related to RWC (%). ♦ and Δ represent pre- and post-flowering dehydrated/rehydrated treatments, respectively.

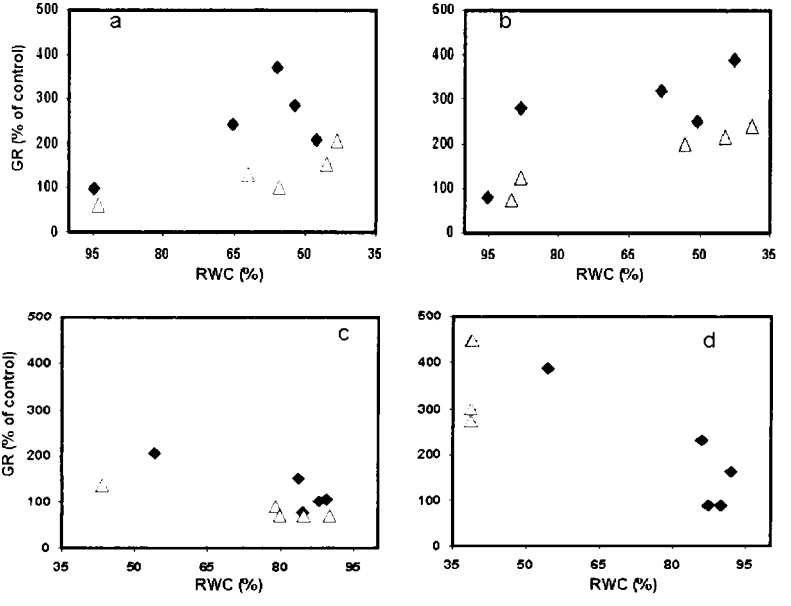

Fig. 2. Glutathione reductase activities (% of control) in maize (a, c) and sorghum (b, d) during pre- and post-flowering dehydration (a, b) and rehydration (c, d) as related to RWC (%). ♦ and Δ represent pre- and post-flowering dehydrated/rehydrated treatments, respectively

In both maize and sorghum, after attaining full recovery following pre-flowering rehydration, the GR activity was enhanced again and peaked at RWCs of approximately 90% (Fig. 2c and d). During post-flowering rehydration, the GR activity in maize was fully restored to the control level, whereas in sorghum the induction of GR activity was observed as rehydration progressed.

It was found that dehydration increased CAT activity in both species (Fig. 3). There were differences between the species with respect to the dehydration treatment. In maize, the changes in CAT activities followed a similar trend during both pre- and post-flowering dehydration. In both cases, the activity increased until the RWC reached approximately 55% and was followed thereafter by a sharp decrease, which was greater for post- than for pre-flowering dehydration.

In sorghum, as water became limiting there was a corresponding increase in CAT activity during both pre- and post-flowering dehydration. CAT activity was maximal at RWCs of 51% and 53% during pre- and post-flowering dehydration, respectively. During pre-flowering dehydration, as the RWC decreased from 51% to 43%, CAT activity was down-regulated from 292% to 192% relative to the control.

The activities of CAT in maize and sorghum dehydrated during the pre-flowering stage returned to the control level at RWCs of 84% and 87%, respectively, but upon further rehydration the activity increased again and peaked at RWCs of 88% and 93%, respectively (Fig. 3c and d). Maize leaves undergoing post-flowering rehydration fully recovered on rehydration, whereas those of sorghum showed only 50% recovery.

Decreases in RWC also induced an increase in APX activity in both maize and sorghum during pre- and post-flowering dehydration, the increase being greater in sorghum during post-flowering dehydration (Fig. 4). In maize plants undergoing pre-flowering dehydration, as the intensity of dehydration became more severe, the APX activity increased continually and reached a maximum of 298% at a RWC of 48%. In plants undergoing post-flowering dehydration, however, a marked increase in activity was noticed until RWC reached 55%, after which there was a decrease. In contrast to maize, the APX activity in sorghum reached a maximum of 233% and 397% at RWCs of 51% and 45% during pre- and post-flowering dehydration, respectively.

In maize plants subjected to pre-flowering dehydration, the APX activities fully recovered to the control level following rehydration, whereas in sorghum, after an initial recovery, the APX activities were reduced as rehydration progressed. Between RWCs of approximately 48% and 80% an induction of APX activities was observed in maize plants undergoing post-flowering rehydration, after which there was a marked decline with a further increase in RWC, though this still represented 3-fold higher activities than in the control plants (Fig. 4c). Sorghum plants, on the other hand, achieved only 50% recovery in APX activities following post-flowering rehydration (Fig. 4d).

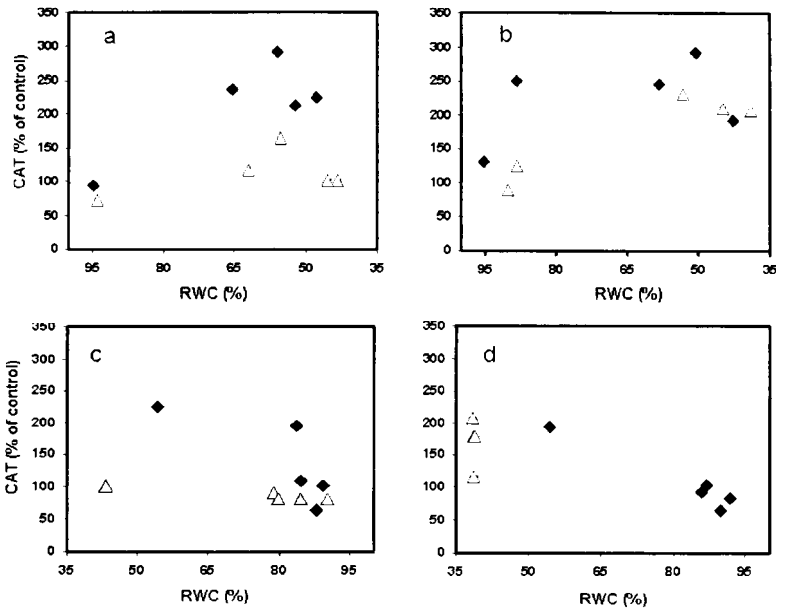

Fig. 3. Catalase activities (% of control) in maize (a, c) and sorghum (b, d) during pre- and post-flowering dehydration (a, b) and rehydration (c, d) as related to RWC (%). ♦ and Δ represent pre- and post-flowering dehydrated/rehydrated treatments, respectively

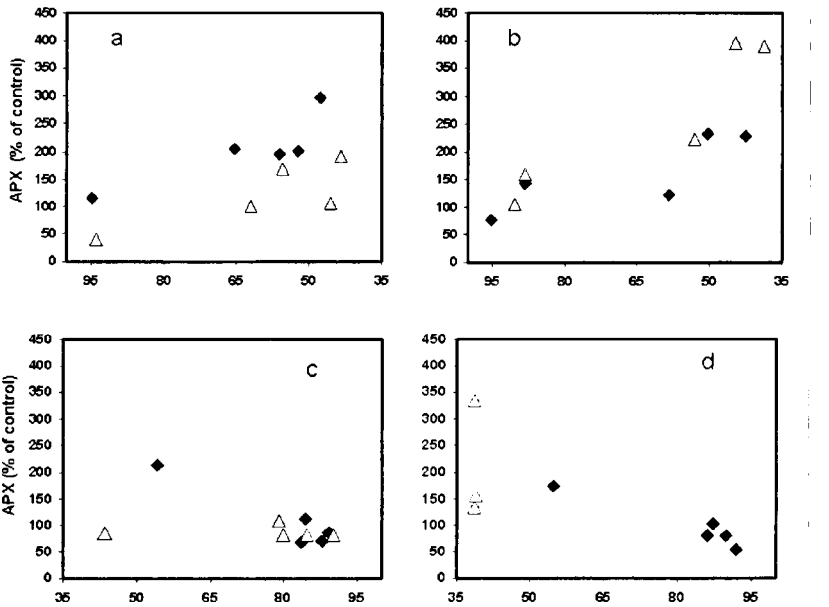

Fig. 4. Ascorbate peroxidase activities (% of control) in maize (a, c) and sorghum (b, d) during pre- and post-flowering dehydration (a, b) and rehydration (c, d) as related to RWC (%). ♦ and Δ represent pre- and post-flowering dehydrated/rehydrated treatments, respectively

Dehydration during the pre- and post-flowering stages led to a marked increase in the MDA content in both maize and sorghum (Fig. 5). Considering the absolute values, the MDA contents were consistently higher in maize than in sorghum under both control and dehydration conditions during the pre- and post-flowering stages. The values ranged from 1.7–4.2, 2.5–5.2 and 2.3–6.4 in maize and from 0.4–1.8, 0.7–3.2 and 1.6–3.0 in sorghum under control, pre-flowering dehydration and post-flowering dehydration conditions, respectively. However, relative to their respective control plants, the increase in MDA content was more marked in sorghum than in maize (Fig. 5a and b). There was a difference in the response of MDA content to dehydration with plant age, with greater increases during pre- than post-flowering dehydration in both species. During pre-flowering dehydration, the MDA content increased by 188% and 613% in maize and sorghum, respectively, while during post-flowering dehydration, the MDA content of maize ranged from 153%–163% and that of sorghum increased to 296%.

In maize leaves undergoing pre-flowering rehydration, there was approximately 55% less MDA than in the control leaves, while in sorghum the MDA contents returned to the control level following pre-flowering rehydration, suggesting that the tissues damaged by ROS were repaired (Fig. 5c and d). During post-flowering rehydration, there were approximately 50% and 65% lower MDA contents in maize and sorghum leaves as compared with the control level.

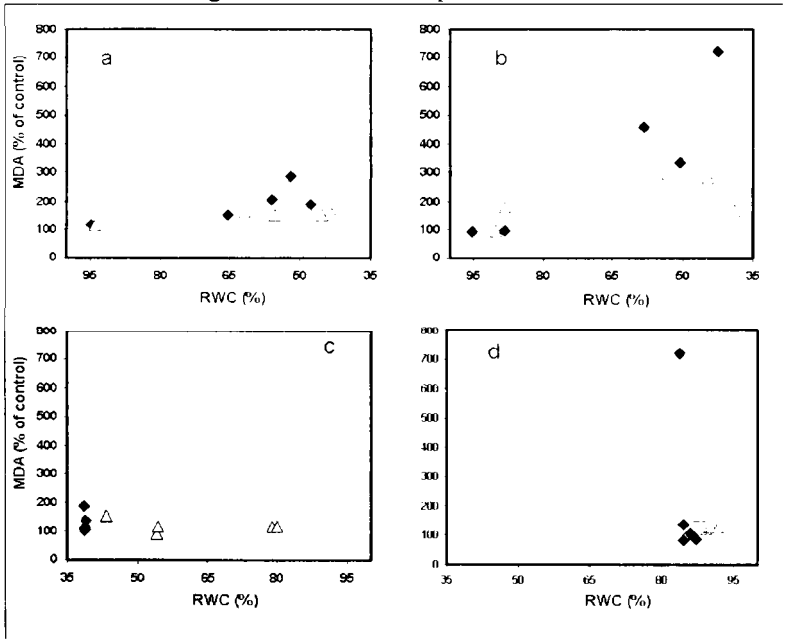

Fig. 5. Malondialdehyde contents (% of control) in maize (a, c) and sorghum (b, d) during pre- and post-flowering dehydration (a, b) and rehydration (c, d) as related to RWC (%). ♦ and Δ represent pre- and post-flowering dehydrated/rehydrated treatments, respectively

Discussion

Antioxidant responses in maize and sorghum during pre- and post-flowering dehydration

SOD plays a central role in the defence against oxidative stress by catalysing the conversion of O_2^- to H_2O_2 and O_2 (Menezes-Benavente and Teixeira, 2004). The enhancement of SOD activity suggests its involvement in the detoxification process of O_2^- in both maize and sorghum in response to pre- and post-flowering dehydration, indicating SOD may function as a ROS scavenger by converting O_2^- to H_2O_2 (Alscher et al., 2002). Many reports indicate that comparatively higher antioxidant activity is positively correlated with tolerant cultivars rather than sensitive ones (Sairam et al., 2002; Reddy et al., 2004; Turkan et al., 2005). Accordingly, the higher dehydration-induced SOD activity in sorghum than in maize may indicate the more important role of this enzyme in the acclimation or adaptation of sorghum to dehydration than in maize during both developmental stages (Fig. 1). Sorghum exhibited much higher SOD activity during post- than pre-flowering dehydration, indicating that the contribution of SOD activity increased with crop age. The findings of this study are in agreement with those of Zhang and Kirkham (1996) and Pastori and Trippi (1993), who observed an increase in SOD in sunflower and sorghum, and in maize, respectively. Although an increase in SOD activity is observed in both species, it might not be the only protective system against lipid peroxidation, because it catalyses the dismutation of O_2^- to H_2O_2, which is also a ROS. This ROS then has to be eliminated by other antioxidant enzymes such as GR, CAT or APX.

GR is the key enzyme in the ascorbate-glutathione cycle, maintaining the GSH:GSSG ratio required for the regeneration of ascorbate, and is also involved in scavenging the products of oxidative stress, such as H_2O_2 (Bartoli et al., 1999). The elevated levels of GR in both maize and sorghum during both developmental stages (Fig. 2) suggest that GR could be a potential mechanism for acclimation or adaptation to dehydration stress by minimizing oxidative stress through the detoxification of ROS and the maintenance of a high GSH:GSSH ratio. The results of this study are in accordance with those of Zhang and Kirkham (1996), who observed increases in the activity of this enzyme in sunflower and sorghum. The later increase in GR could be owing to accelerated senescence induced by dehydration in maize during both pre- and post-flowering and in sorghum during post-flowering dehydration.

Along with SOD, CAT constitutes a front-line defence against ROS, converting H_2O_2 to water (Menezes-Benavente and Teixeira, 2004). The results indicated that the activity of CAT increased above the control in both species in response to dehydration during both the pre- and post-flowering stages (Fig. 3a and b). This has also been reported in other crop species exposed to oxidative stresses, such as rice (Vaidyanathan et al., 2003), tobacco (Van Rensburg and

Kruger, 1994), cotton (Rajguru et al., 1999) and sugar beet (Bor et al., 2003). The higher level of CAT activity during pre- than post-flowering dehydration may indicate a greater contribution to the decomposition of H_2O_2. The decrease in CAT activity in maize during the late phases of pre- and post-flowering dehydration (Fig. 3a) could be the result of genetic differences. The enhanced activity of CAT observed in sorghum during both pre- and post-flowering dehydration compared with maize aids in the rapid elimination of the H_2O_2 which could have been produced during the two developmental stages. Confirming the results of the present work, an increase in CAT activity under drought stress conditions was reported by Jagtap and Bharagava (1995) in sorghum and Van Rensburg and Kruger (1994) in tobacco.

The enzyme APX, located in the cytosol and chloroplasts, breaks down H_2O_2 efficiently using ascorbate as the electron donor (Bor et al., 2003). Since the increased activity of SOD during dehydration in both the pre- and post-flowering stages was accompanied by an increase in APX activity in the leaves of both species (Fig. 4a and b), it is suggested that SOD and APX work more efficiently in concert to scavenge ROS such as O_2^- and H_2O_2, which might possibly be produced during dehydration. Similar results were reported in other crop species such as wheat (Bartoli et al., 1999; Lascano et al., 2001), tobacco (Van Rensburg and Kruger, 1994), cotton (Meloni et al., 2003) and rice (Vaidyanathan et al., 2003). The role of APX appears to be equally important irrespective of the crop age in maize, whereas higher activity was exhibited during post- than pre-flowering dehydration in sorghum (Fig. 4a and b), suggesting a greater contributory role in the former case.

These collective results suggested that the ROS formed in maize and sorghum during pre- and post-flowering dehydration were detoxified by the increased activities of antioxidant enzymes (SOD, GR, CAT and APX) (Figs. 1–4). In sorghum, there seems to be a co-ordinated response of all antioxidant enzymes in eliminating ROS, as evidenced from the higher increases during both pre- and post-flowering dehydration and the lower level of MDA, as compared to maize (Fig. 5). In maize, although there was an increase in antioxidant enzymes, it appeared to be greatly dependent on SOD (Fig. 1a) and APX (Fig. 4a), as evidenced by the decrease in the activities of GR (Fig. 2a) and CAT (Fig. 3a) with an increase in the intensity of dehydration during both pre- and post-flowering dehydration.

Lipid peroxidation, measured as MDA content, was used in both maize and sorghum as an indication of ROS damage due to dehydration. The higher MDA content observed in maize than in sorghum (Fig. 5) indicates the prevalence of ROS in maize tissue compared with sorghum. In the current study it was found that, in spite of a consistent increase in the activities of antioxidant enzymes, particularly SOD and APX, maize exhibited higher MDA levels during both pre- and post-flowering dehydration. This indicated that the increases in these antioxidant enzyme activities could not provide sufficient

protection to the membrane against ROS. It is possible that the extensive senescence processes observed in maize suppressed the antioxidant defence systems due to diminished activities of these antioxidants and caused exposure of the tissues and cells to more oxidative stress. Sorghum, on the other hand, exhibited enhanced enzyme activities (SOD, GR, CAT and APX) throughout the pre- and post-flowering dehydration periods. The coordinated action of these enzymatic antioxidants in sorghum might have contributed to providing effective protection against oxidative stress, minimizing lipid peroxidation under dehydration conditions better than in maize. This suggests that sorghum has a higher hereditary and induced antioxidant capacity under dehydration, which provides it with better protection from oxidative damage. Parallel to these results, low MDA content was found to be important for abiotic stress tolerance by other authors. Azevedo Neto et al. (2006) reported a low level of MDA in a salt-tolerant maize genotype and a high level in a salt-sensitive genotype. Similar results correlating lipid peroxidation with antioxidative system activity were also reported by other researchers (Hernández and Almansa, 2002; Koca et al., 2007).

Antioxidant responses in maize and sorghum during pre- and post-flowering rehydration

Enzymatic antioxidants (SOD, GR, CAT and APX) appear to have an important role for the recovery of both maize and sorghum in providing additional protection against ROS during pre- and post-flowering rehydration. The extent of their protective role varied, however, depending on the developmental stage and species. The increase in SOD, GR, CAT and APX activities during the late phase of rehydration following pre-flowering dehydration in sorghum and the retention of elevated levels of these enzymes during post-flowering rehydration compared with the decrease in the activities of SOD and APX during pre-flowering rehydration and in those of GR and CAT during post-flowering rehydration in maize, relative to their respective control plants (Figs. 1c and d, 4c and d), may suggest that these enzymes operate better in sorghum than in maize. In sorghum, ROS might be detoxified more efficiently, thereby lowering the exposure of membrane lipids to high ROS. The increase in GR and CAT activities during the late phase of pre-flowering rehydration in maize may indicate that the operation of these enzymes was more efficient when maize was exposed to moderate stress conditions. On the other hand, the retention of high SOD and APX activities during post-flowering rehydration may be viewed as a key factor in controlling the oxidative stress that might have occurred during the rehydration process and in providing additional protection against ROS. The involvement of enzymatic antioxidants in scavenging ROS was also revealed in other crop species such as spinach (Tanaka et al., 1985) and cotton (Foster and Hess, 1980).

In maize, where SOD and APX decreased to the control level during pre-flowering rehydration and GR and CAT during post-flowering rehydration, the ROS formed at both developmental stages were able to cause membrane damage.

Conclusions

The present study revealed that the exposure of maize and sorghum to pre- and post-flowering dehydration resulted in an increase in the activities of the enzymatic antioxidants involved in the detoxification of reactive oxygen species. Under both pre- and post-flowering dehydration conditions, sorghum exhibited consistent and comparatively higher increases in all enzymatic antioxidant activities than maize, thus minimizing the extent of lipid peroxidation (as determined by malondialdehyde content). In maize, with the exception of superoxide dismutase and ascorbate peroxidase, the activities of other enzymatic antioxidants (glutathione reductase and catalase) declined with an increase in the intensity of pre- and post-flowering dehydration, leading to higher amounts of malondialdehyde. Since sorghum exhibited comparatively higher overall activities of enzymatic antioxidants during both pre- and post-flowering dehydration, selection based on these criteria may help in the development of genotypes tolerant to dehydration. However, several other antioxidants, which were not measured in the present study and could eliminate reactive oxygen species or limit dehydration-induced damage in both species, will require further study.

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RELATIONSHIP BETWEEN S-METHYLMETHIONINE TREATMENT AND THE ACTIVITIES OF ANTIOXIDANT ENZYMES IN MAIZE (*Zea mays* L.) LEAVES AT CHILLING TEMPERATURES

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S-methylmethionine (SMM) is an important intermediary compound in the sulphur metabolism and has been shown to play a possible role in moderating the damaging effects of low temperature stress. The present work investigated the extent to which SMM is capable of influencing the activity of antioxidant enzymes when the subtropical species maize is exposed to chilling temperatures during the early developmental phase. SMM was found to contribute to the protection of maize seedlings against low (<14°C) temperature stress by enhancing the activity of certain antioxidant enzymes to varying extents, and thus helping to neutralise the reactive oxygen species (ROS) formed at this temperature. Results obtained in a gradient plant growth chamber revealed that, with the exception of catalase, SMM increased the activity of all the antioxidants studied (glutathione reductase, glutathione-S-transferase, guaiacol peroxidase, ascorbate peroxidase), particularly in the lower ranges of the temperature gradient (6–14°C).

Key words: S-methylmethionine, antioxidant enzymes, chilling temperature, maize

Abbreviations: CAT = catalase, GR = glutathione reductase, APX = ascorbate peroxidase, GST = glutathione-S-transferase, POD = guaiacol peroxidase, SMM = S-methylmethionine, ROS = reactive oxygen species

Introduction

SMM is a naturally occurring, biologically active compound, which is becoming increasingly recognised as an important component in the plant S metabolism. Its occurrence in the plant kingdom is now thought to be general (Szegő et al., 2007).

An increasing body of data has been published in recent years on the functions of SMM, showing that it is synthesised from methionine (Met), plays a role in the regulation of Met and S-adenosyl-methionine (AdoMet) levels (Pimenta et al., 1998; Kocsis et al., 2003) and is involved in the methylation processes taking place in cells (Ranocha et al., 2000).

SMM may also play an outstanding part in overcoming the damaging effects of cold stress. This was suggested by the characteristically high SMM content in members of the Brassicaceae family, which has a role in tolerance to cold stress (Gyetvai et al., 2002; Rácz et al., 2008). In cold-sensitive plants, SMM treatment may lead to an increase in tolerance. For example, an improvement in photosynthetic activity was recorded in cold-treated maize when SMM was applied. The favourable effect of SMM was also demonstrated by studies in which the increased permeability of membranes and the consequent ion leakage from the cells was investigated in response to cold stress (Lásztity et al., 1997; Gyetvai et al., 2002; Rácz et al., 2008). The effect of externally applied SMM on the synthesis of polyamines may be related in part to the regulation of the AdoMet level, since the decarboxylated derivative of AdoMet supplies the propylamino group involved in the formation of spermidine from putrescine and of spermine from spermidine. The wide-ranging effect of polyamines on plant life processes also accounts for the complex role of SMM. The ability of polyamines to stabilise membranes, proteins and DNA may also be related to the mechanism by which SMM overcomes stress (Galston and Kaur-Sawhney, 1995; Bouchereau et al., 1999).

One of the main causes of the damaging effect of low temperature is the accumulation of reactive oxygen species (ROS) (Prasad et al., 1994), so the response of the antioxidant system is of key importance in protection against cold stress, especially for a subtropical species such as maize. The antioxidant system consists of both enzymatic and non-enzymatic components. The non-enzymatic components are water- or lipid-soluble compounds with antioxidant properties, and some of the enzymatic components (APX, GR) participate in the elimination of ROS by catalysing the reactions or regeneration of these compounds. The most important enzymatic antioxidants can be divided into several major systems which can be found at sites where ROS are formed. These include the superoxide dismutases, the water–water cycle, the ascorbate–glutathione cycle, the catalases and the glutathione–peroxidase cycle (Mittler, 2002). Based on recent results (Szegő et al., 2009), in the present work the effect of SMM on the activity of various enzymatic antioxidant components was studied during chilling stress in the early developmental phase of maize.

Materials and methods

Plant material

Seeds of maize (*Zea mays* L., hybrid Norma) were disinfected in 5% sodium hypochlorite solution for 5 min, then rinsed several times in distilled water. They were then germinated between moist filter papers in a germinating cabinet (type: G 30, Conviron, Canada) at 25°C in the dark for 72 h. The seedlings were then placed on a stainless steel mesh (5–7 seedlings/pot) with their roots in Hoagland nutrient medium with the following composition: macronutrients: 0.3125 mM KNO_3, 0.45 mM $Ca(NO_3)_2$, 0.0625 mM KH_2PO_4, 0.125 mM $MgSO_4.7H_2O$; micronutrients: 11.92 µM HBO_3, 4.57 µM $MnCl_2.4H_2O$, 0.191 µM $ZnSO_4.7H_2O$, 0.08 µM $CuSO_4.5H_2O$, 0.024 µM $(NH_4)_6Mo_7O_{24}.4H_2O$, 15.02 µM $FeSO_4.7H_2O$, 23.04 µM $Na_2EDTA.5H_2O$. The plants were raised

for 21 days (until the third leaf was completely developed) in a PGV-36 growth chamber (Conviron, Canada) with 16 h light/8 h dark, day/night temperatures of 22/20°C, light intensity of 340 μmol m^{-2} s^{-1} at leaf level and 70% relative humidity. The nutrient solution was changed every two days. SMM treatment was carried out on the 22nd day, by adding 0.01% SMM to the medium. Control plants continued to grow on SMM-free medium. After 1 day the SMM-containing medium was exchanged for normal nutrient solution and the plants were transferred to the gradient chamber (Tischner and Veisz, 1996) for low temperature treatment. Apart from the temperature, the growth parameters were the same as those detailed above. In the first experiment chilling was carried out at 5°C, while in the second there was a temperature gradient of 6–8–10–12–14°C. Samples for the determination of enzyme activity were taken from the fully developed third leaf after 1, 4 and 6 days of chilling in Experiment 1 and after 4 days in the gradient experiment.

Determination of enzyme activities

During isolation 0.5 g plant material was ground on quartz sand in a mortar with 2.5 ml ice-cold 0.5 mM Tris buffer (pH 7.4) containing 3 mM $MgCl_2$ and 1 mM EDTA. The homogenate was centrifuged at 15,000 *g* for 20 min at 4°C. The supernatant was transferred to Eppendorf tubes and the total protein concentration was determined with Bio-Rad reagent, as proposed by Bradford (1976), recording the absorbance of the reaction mixture spectrophotometrically at 595 nm.

Glutathione reductase (EC 1.6.4.2)

The activity of glutathione reductase (GR) was measured in fresh supernatant and that of the other enzymes after freezing at –20°C. The enzyme activities were determined photometrically (UV-VIS 160A, Shimadzu, Japan). The samples were kept on ice until measurement, but the measurements were made at room temperature. The enzyme activities were expressed as the change in absorbance (ΔA min^{-1} g^{-1} protein).

Guaiacol peroxidase (EC 1.11.1.7)

The activity of guaiacol peroxidase (POD) was recorded spectrophotometrically as the increase in absorbance at 470 nm as the result of guaiacol oxidation (Ádám et al., 1995). The reaction was initiated by adding hydrogen peroxide solution. The reaction mixture consisted of 88 mM Na acetate buffer (pH 5.5), 0.88 mM guaiacol, 0.0375% H_2O_2 and 50 μl plant sample in a total volume of 3 ml.

Ascorbate peroxidase (EC 1.11.1.11)

The activity of ascorbate peroxidase (APX) was determined in a 2.25 ml reaction mixture containing 0.2 M Tris buffer (pH 7.8), 5.625 mM ascorbic acid, 0.042% H_2O_2 and 50 μl plant sample. The reaction was initiated by adding the H_2O_2. The decline in ascorbic acid was monitored at 290 nm (Janda et al., 1999).

Glutathione reductase (EC 1.6.4.2)

The activity of glutathione reductase was recorded by measuring the reduction of 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) at 412 nm, as described by Smith et al. (1988). The reaction mixture consisted of 75 mM Na-phosphate buffer (pH 7.5), 0.15 mM diethylene triamine pentaacetic acid, 0.75 mM DTNB, 0.1 mM NADPH and 1 mM GSSG in a total volume of 1 ml. The reaction was initiated by adding 50 μl plant sample.

Glutathione-S-transferase (EC 2.5.1.18)

The activity of glutathione-S-transferase (GST) was determined spectrophotometrically at 340 nm using the method of Mannervik and Guthenberg (1981), by monitoring the formation of S-2,4-dinitrophenyl glutathione. The 2.75 ml reaction mixture contained 72.5 mM Na-phosphate buffer (pH 6.5), 2.6 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene, and the reaction was initiated by adding 100 μl plant sample.

All the chemicals were of analytical quality and were purchased from Sigma and Merck.

Results

The first experiment aimed to determine whether antioxidant enzymes were activated during chilling at 5°C and whether there was any difference between control and SMM-treated plants in this respect. For this purpose, the activity of the enzymes GR, GST, CAT, APX and POD was measured in leaf samples taken on the 1st, 4th and 6th days of chilling treatment.

It was found that all the antioxidant enzymes tested were activated in response to 5°C chilling treatment, but to different extents and at different rates (Fig. 1). The activity of GR, GST and CAT reached a maximum on the first day. The increase was approx. 2-fold for GR and CAT, but only around 1.5-fold for GST. The activity of CAT began to decline from the 4th day, while that of the other two enzymes remained high until the 6th day. The APX activity rose very steeply in response to chilling stress, doubling on the first day, and reaching a maximum (5-fold) on the 4th day. POD exhibited no change in activity on the first day, and only increased slightly, but not significantly, on the 4th day.

Differences were noted in both the rate and extent of activation of the individual enzymes as the result of SMM treatment. In the course of chilling the activity of the GR, GST and CAT enzymes was much the same in the control and SMM-treated plants on the first day, but in treated plants the activity continued to rise until the 4th day, when it was significantly greater than in control plants. By the 6th day, however, the activity of all three enzymes declined (that of catalase to the greatest extent) in SMM-treated plants. APX was activated to a greater extent in SMM-treated plants even on the first day, when the enzyme activity was more than doubled. Similarly high activity could still be observed on the 4th day, but by the 6th day it had declined, so there was less difference between the control and treated plants. The POD activity increased earlier after SMM treatment than in the control, exhibiting a substantial rise even on the first day. Despite a decrease during the long chilling period, it was still significantly greater than in the control plants on the 6th day (Fig. 1).

The results indicated that the activity of GR, GST and CAT was greatest on the 4th day of chilling treatment in SMM-treated plants. In the case of APX the rate of activation was accelerated by SMM, while the SMM treatment had a positive effect on both the rate and extent of activation for POD.

In the second experiment the effect of SMM on the antioxidant enzyme system in maize seedlings was investigated over a wider chilling temperature range (14–12–10–8–6°C) in a gradient chamber (Fig. 2). Measurements of enzyme activities were carried out on the 4th day of treatment. The activity of the GR enzyme began to increase in control plants at 10°C, reaching a maximum at 8°C. As the result of SMM treatment the activation of the enzyme was first observed at 8°C, becoming stronger at 6°C, when it significantly exceeded the values recorded in control plants. The activity of glutathione-S-transferase was somewhat higher in control plants than in SMM-treated plants at 10°C, while at

6°C it decreased in the control. At this temperature, however, the effect of SMM was pronounced; the GST activity in treated plants was significantly higher at 6°C compared with both the control plants and with SMM-treated plants at 10°C. The CAT activity rose in response to chilling in both the control and the SMM-treated plants, the highest values being recorded at 10°C in the control and at 6°C after SMM treatment. The APX activity in control plants did not change significantly at the various chilling temperatures, whereas in response to SMM treatment significantly higher values of enzyme activity were recorded at all the temperatures. The greatest activity was observed at 8°C. The POD activity also exhibited little change as a function of temperature in the control plants, while in response to SMM the maximum value was reached at 6°C. This was greater than the activity recorded in control plants at this temperature (Fig. 2).

It could be concluded from the results that the effect of SMM treatment was primarily felt at lower temperatures: at 6°C it enhanced the activity of GR, GST and POD compared with the control. In the case of APX and CAT the ability of SMM to stimulate enzyme activity could be detected at all the temperatures on the chilling gradient, but for CAT the effect was again strongest at the lowest (6°C) temperature.

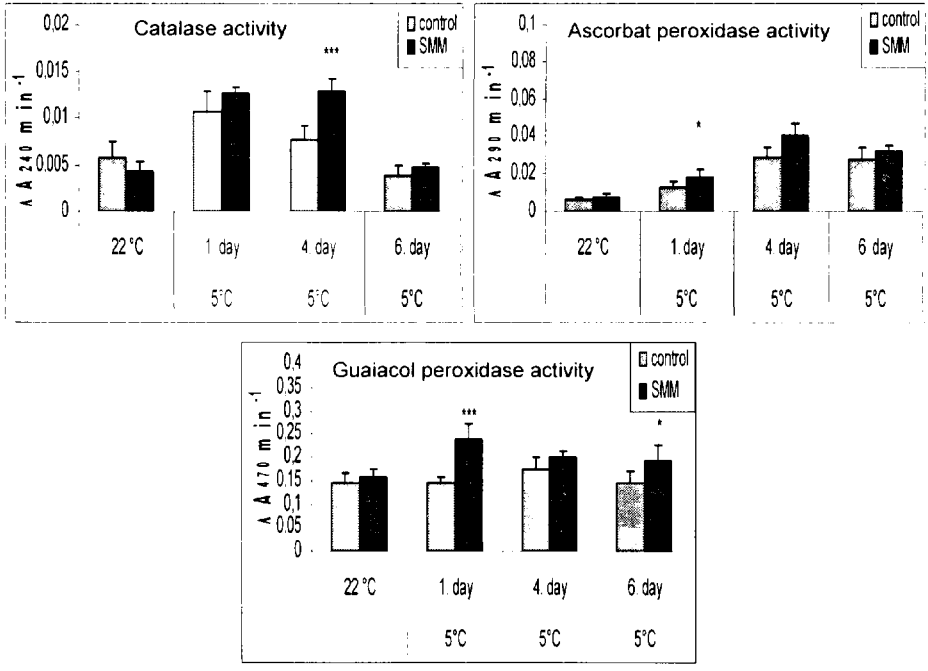

Fig. 1. Effect of chilling treatment (5°C, 1, 4, 6 days) on the activity of antioxidant enzymes (GR, GST, CAT, APX, POD) in control and SMM-treated maize seedlings

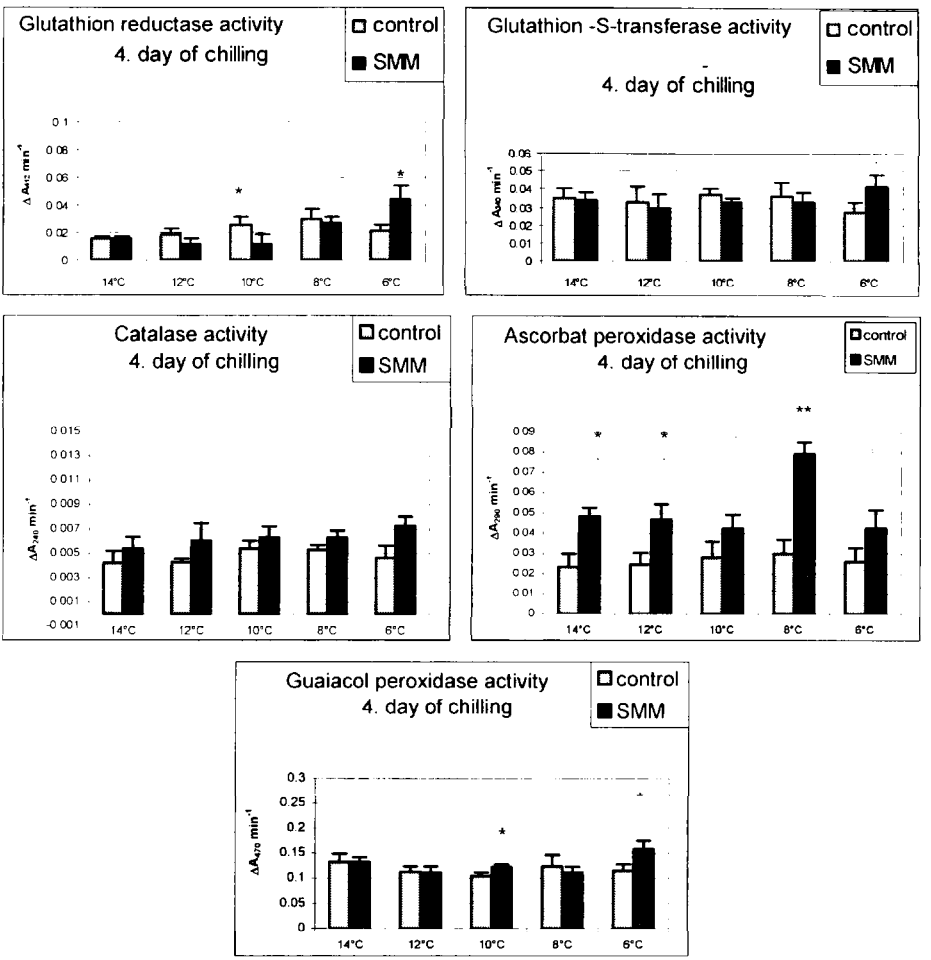

Fig. 2. Effect of a low temperature gradient (4 days, 14–6°C) on the activity of antioxidant enzymes (GR, GST, CAT, APX, POD) in control and SMM-treated maize seedlings

Discussion

The development of stress tolerance in plants is related to a rise in the activity of antioxidant systems. The increased ROS level induces the up-regulation of antioxidant enzyme activity, thus protecting the plants from the damaging effects of oxidative stress (Davey et al., 2000). This increase in the activity of the antioxidant defence system against environmental stress factors develops over a long period in a process known as adaptation. A large number of species of subtropical origin, such as maize, rice, tomato, paprika, marrow, cucumber, etc., are now grown in the temperate zone, but the conditions are sub-optimal and these species did not originally possess defence mechanisms against

low temperatures (Janda et al., 2005). Breeders must thus select genotypes with an adequate level of tolerance, in order to develop tolerant varieties. Depending on the species, this process may be very time-consuming. Over the last decade increasing attention has been paid to compounds involved in the metabolism, which could be applied exogenously to stimulate the antioxidant systems. These compounds include polyamines (Zhang et al., 2009), salicylic acid and its derivatives (Janda et al., 1999; Kang et al., 2003; Wang and Li, 2006), methyl jasmonate (Cao et al., 2009) and SMM, the compound investigated in the present work (Gyetvai et al., 2002; Rácz et al., 2008; Szegő et al., 2009). SMM regulates the methionine and S-adenosyl methionine (AdoMet) levels and the sulphur transport, and is the starting compound for the synthesis of dimethyl-sulphoniopropionate, the most powerful plant osmoprotectant yet discovered (Hanson et al., 1994). SMM protects the photosynthetic apparatus, especially the PSII system, and is transformed during the synthesis of spermidine, which ensures the integrity of cell membranes (Rácz et al., 2008; Szegő et al., 2009). The data suggest that SMM causes a rise in the activity of certain antioxidant enzymes, thus contributing to the elimination of ROS. A number of authors have emphasised the role of APX and GR in reducing the H_2O_2 level (Noctor and Foyer, 1998). In the absence of CAT activity, the H_2O_2 arising in the chloroplasts is neutralised by the ascorbate–glutathione cycle with the help of APX. The oxidised glutathione formed as the end-product of this process is reduced by GR using NADPH. This is confirmed by the increase in GR and APX activity observed in SMM-treated plants at chilling temperatures. The present results provided further proof of the decisive importance of APX, the activity of which was enhanced in maize leaves by SMM over the whole of the low temperature gradient, thus contributing to the avoidance or mitigation of chilling damage.

It is important to note the complex function of the GST enzyme. As a substrate it utilises glutathione, which acts as a redox state regulator in the cell, so the quantity and availability of the enzyme is of great significance. At the same time, GST also plays an important role in the formation of the complexes transported by means of intracellular transport processes and in detoxification, as it is only when compounds such as anthocyanins or other toxic materials are bound to the glutathione produced by this enzyme that they can be transported and sequestered. GST is thus more than just an antioxidant enzyme and exhibits slightly different behaviour.

Various reviews also indicate that, in the same way as abiotic stress factors, exogenously applied compounds do not have the same effect on all the antioxidant enzymes. This can be clearly demonstrated in the case of polyamines, salicylic acid and SMM. Further extensive studies will be required to obtain a better understanding of the diverse behaviour, i.e. differing activity levels, of antioxidant enzymes under unfavourable conditions.

Acknowledgements

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INDOLE-3-BUTYRIC ACID APPLICATION MITIGATES SODIUM CHLORIDE STRESS IN TWO COTTON CULTIVARS DIFFERING IN SALT TOLERANCE

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Soil salinity is a major constraint to food production because it limits crop yield and restricts the use of land previously uncultivated. Breeding for tolerance to salinity in crops has usually been limited by the lack of reliable traits for selection. The mechanism of salt tolerance in two cotton (*Gossypium barbadens* L.) cultivars (Giza 70 and Giza 88) and their responses to shoot spraying with 200 ppm m^{-3} IBA were studied.

Treatment with IBA not only improved the growth of salt-affected Giza 70, but also increased the growth of this cultivar up to −2.7 MPa and reduced the inhibitory effect of salinity on photosynthetic pigments.

This was accompanied by differences in the accumulation of sucrose and total soluble sugars and in the total available carbohydrate and protein contents. IBA ameliorated the inhibitory effect of salinity on growth, increased the carbohydrate and protein contents of both cotton cultivars and markedly retarded the accumulation of proline and glycine betaine. It resulted in the reduction of Na^+ accumulation in Giza 70, while in Giza 88 it enhanced the absorption and translocation of K^+, resulting in higher K^+/Na^+ ratios in the shoots. There were pronounced differences in the electrophoretic patterns of the proteins in both cultivars under salt stress and IBA treatment.

Key words: indole-3-butyric acid, sodium chloride, salt tolerance, proline, glycine betaine, *Gossypium barbadens*

Introduction

Salinity is one of the major environmental factors limiting crop production (Debez et al., 2006; Koyro, 2006; Kant et al., 2007). The reduction in plant growth is a consequence of several physiological responses, including the modification of water status, photosynthetic efficiency, and carbon allocation and utilization (Nabil and Coudret, 1995; Popova et al., 1996; Romero-Aranda et al., 2006).

Plants have evolved various protective mechanisms that allow them to acclimate to unfavourable saline environments for continued survival and growth. One ubiquitous mechanism is the accumulation of certain organic metabolites of low molecular weight, known as compatible solutes (Bohnert et al., 2001). Proline accumulation might be used as an indicator in selection for withstanding saline stress due to its involvement in osmoregulation (Ueda et al., 2007). Glycine betaine is a quaternary ammonium compound; it is a dipolar but electrically neutral molecule at physiological pH, so it is regarded as a particularly effective compatible solute (Le Rudulier et al., 1984; Huang et al., 2000).

Potassium plays an important role in balancing membrane potential and turgor, activating enzymes, and regulating osmotic pressure, stoma movement and tropisms (Cherel, 2004). To maintain normal cell metabolism, the K^+ content in wheat cells is kept around 150 mM and the Na^+ content at about 30 mM, resulting in a K^+/Na^+ ratio of approximately 5 (Carden et al., 2003). A suitable K^+/Na^+ ratio is important for the adjustment of cell osmoregulation, turgor maintenance, stomatal function, activation of enzymes, protein synthesis, oxidant metabolism and photosynthesis (Shabala et al., 2003).

Several investigators reported that salt stress caused an alteration of gene expression in plants, resulting in the inhibition or enhancement of specific protein synthesis (Chang et al., 1996).

SDS-PAGE analysis of proteins in peanut (*Arachis hypogaea* L.) reveals that plants grown under NaCl-stressed conditions show induction (127 and 52 kDa) or repression (260 and 38 kDa) in the synthesis of a few polypeptides (Hassanein, 1999). In radish (*Raphanus sativus* L.) salt stress causes the accumulation of a 22-kDa protein (p*I* of 7.5) and its mRNA in the leaves (Lopez et al., 1994). Yen et al. (1997) reported the accumulation of five polypeptides, with molecular masses estimated by SDS and 2D-PAGE to be 40, 34, 32, 29 and 14 kDa, in the calli of *M. crystallinum* under NaCl stress. These polypeptides were classified into two distinct groups according to the course of induction: early responsive (40, 34 and 29 kDa) and late responsive (32 and 14 kDa) proteins. NaCl induced the accumulation of four polypeptides with molecular masses of 61, 51, 39 and 29 kDa in maize roots (Tamas et al., 2001). Tuteja (2007) presented the first direct evidence in plants that phospholipase C (PLC) functions as an intercellular effector molecule for the α-subunit of pea heterotrimeric G-protein and regulates its activities, including salinity stress tolerance.

The major effect of salinity in the root environment is reduced hormone delivery from roots to leaves, which could induce an inhibition of crop growth. The growth-promoting substance indole-3-butyric acid (IBA) is used as an auxin of choice for root formation on cuttings (Hartmann et al., 1997). Like the auxin indole-3-acetic acid (IAA), IBA affects lateral root induction and the elongation of roots, shoots and hypocotyls (Zolman et al., 2000; Rashotte et al., 2003). IBA and IAA can be interconverted, which has led to the suggestion that IBA may act as a precursor to IAA (Bartel et al., 2001).

The purpose of this study was to compare the tolerance mechanisms of two cotton cultivars, Giza 70 and Giza 88, to salinity stress, and their behaviour in respect to compatible osmolytes, sucrose, proline and glycine betaine accumulation.

IBA was applied exogenously in order to assess whether it alleviated the effect of salinity on dry matter, tolerance index and some relevant metabolic activities (carbohydrates, proteins). It was also hoped to reveal the effect of IBA on changes in protein profiles under salinized conditions.

Materials and methods

The seed of cotton cultivars was obtained from the breeding programme of the Agricultural Research Centre, Dokky, Cairo, Egypt.

Experimental conditions

The experiment on *Gossypium barbadens* cvs Giza 70 and Giza 88 was repeated three times in a growth chamber programmed for an 18/6 h light/dark cycle, temperatures of 20–24°C during the day and 15–19°C during the night and relative humidity of 75% (to simulate field conditions). Five seeds of one cotton cultivar were sown in each pot (15 cm in diameter), and a week later the plants were thinned to three per pot. Each treatment consisted of ten pots. The pots were irrigated to 90% of the water-holding capacity, with NaCl salinization levels corresponding to osmotic potentials of 0, −0.3, −0.6, −0.9, −1.8, −2.4 and −2.7 MPa. The pots in each treatment were moved around the growth chamber every other day to make the conditions more uniform. At the age of three weeks the ten pots in each salinized and control treatment were divided into two groups, one of which was sprayed three times with 1 mM IBA solution at 5-day intervals, while the others were not sprayed. The plants were irrigated daily with water to reach the above salinization levels.

At the end of the experimental period (45 days), the plants were rinsed with deionized water prior to collection and dissected into roots and stems. The photosynthetic pigment contents in the leaves were determined as described by Moran (1982). The dry matter of the roots and stems was determined after drying the freshly harvested organs in an aerated oven at 80°C to constant weight. The tolerance index (TI) was calculated according to De La Rosa-Ibarra and Maiti (1995).

Reducing sugars (RS), total soluble sugars (TSS) and total carbohydrate contents (TCC) were determined according to Naguib (1964). Sucrose was determined enzymatically after hydrolysis with 0.1% invertase, while protein contents were determined by the method of Bradford (1976). Proline content was estimated according to Bates et al. (1973) and glycine betaine by the method of Grieve and Grattan (1983). The mixed acid digestion method of Allen et al. (1974) was used to prepare samples for the determination of minerals. Approximately 0.2 g of oven-dry, finely powdered plant material was mixed in a 50-ml Kjeldahl flask with 1 ml of 60% perchloric acid, 5 ml conc. nitric acid and 0.5 ml conc. sulphuric acid. The sample was heated slowly at moderate heat until the disappearance of white fumes, then strongly until the mixture turned colourless. When cool the solution was made up to volume (25 ml) and kept for the determination of metals.

The sodium, potassium and calcium concentrations were estimated using a flame photometer (CORN NG400, USA). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDA-PAGE) was carried out using the discontinuous buffer system described by Laemmli (1970) and modified by Hames and Rickwood (1990).

Statistical analysis

All statistical analyses were conducted using SAS, version 8.1. The experiments were arranged in a completely randomized design with three replications. Three cotton samples were collected per treatment. T-tests at $P = 0.05$ were carried out using the pooled mean square from the ANOVA. Differences between means were analysed by Duncan's new multiple range test, followed by ANOVA at $P<0.05$.

Results

The dry matter and tolerance index of the roots and stems of the two cotton cultivars differed in their response to salinity stress (Fig. 1). Giza 70 and Giza 88 tolerated salinity up to a level of –2.4 MPa and –2.7 MPa NaCl salinity, respectively. The inhibitory effect of salinity on dry matter and tolerance index was more pronounced in Giza 70 than in Giza 88. However, there were significant differences between the two cotton cultivars at high salinity levels (–2.4 MPa), with reductions in the dry matter and consequently the tolerance index of the roots (about 0.20 and 0.47) and stems (0.41 and 0.48) of Giza 70 and Giza 88, respectively, as compared with the control. Also, Giza 88 tolerated salinity up to –1.8 MPa, while lower salinity levels stimulated the growth parameters. Spraying the shoots with 200 ppm m^{-3} IBA alleviated the inhibitory effect of salinity on the dry matter yield and increased the investigated parameters compared with those of the unsalinized plants. At –2.4 MPa the decrease in dry matter in the roots of Giza 70 and Giza 88 was 80% and 52.5%, respectively. The corresponding values were 71% and 45%, respectively, after spraying with IBA.

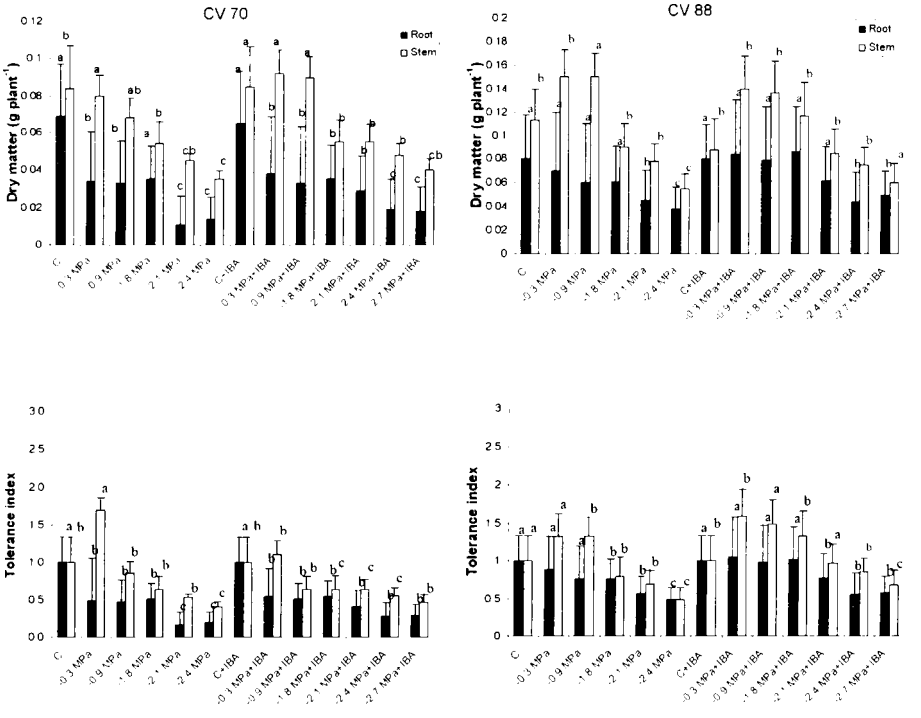

Fig. 1. Effect of salinity and IBA treatment on the dry matter content (g $plant^{-1}$) and tolerance index (TI) of roots and stems of the cotton cultivars Giza 70 and Giza 88. Data are presented as means ± SE (n=3). Different letters above the bars indicate significant differences at $p<0.05$ according to Duncan's new multiple range test

Increasing salinity levels in the growing medium of Giza 70 and Giza 88 cotton plants resulted in a significant decrease in chl.a and chl.b, particularly in Giza 70, while the change in the carotenoids content was insignificant. There was a marked increase in the carot/(chl.a+chl.b) ratio, particularly in Giza 70, with increasing salinity levels (Fig. 2), indicating the role of carotenoids in the photoprotection of photosynthetic pigments under stress conditions. Treatment with IBA mitigated the inhibitory effect of salinity stress to some extent, especially in Giza 70. At –2.4 MPa the chl.a content was 33% compared to the control, while this rose to 60% after IBA treatment. Thus, IBA induced the biosynthesis of chl.a.

The effect of salinity on the carbohydrate and protein contents varied according to the organ and the salinity levels. In the root system of cotton cultivars, there were no significant differences in reducing sugars between the control and salinity treatments at low and moderate salinity levels, while there was a significant reduction at higher salinity levels. Sucrose, however, significantly increased in the stems of both cotton cultivars, but decreased in the roots at high salinity levels (Fig. 3). Although the total available carbohydrates (TAC) contents in both the roots and stems of both cultivars were significantly decreased by increasing NaCl concentrations, the TSS/TAC ratios were greatly increased, particularly in Giza 70.

The data in Figure 4 show that the soluble protein content increased significantly in the stems up to a level of –0.9 MPa and insignificantly increased in the roots up to –1.8 MPa, after which a decrease was recorded for Giza 70. There was a significant reduction in the insoluble and hence in the total protein in the roots and stems of both cultivars in response to salinity stress. Spraying the salinized plants with IBA generally resulted in higher carbohydrate and protein contents than in unsprayed plants. This was also true for the IBA-treated control.

The proline content increased significantly with increasing soil salinity levels in both the roots and stems of Giza 70. In the stems of Giza 88 proline accumulated up to the –1.8 MPa level, after which it gradually decreased.

There was a gradual increase in glycine betaine in the roots and stems of both cultivars with increasing salinity up to –2.4 MPa (Fig. 5). The accumulation of glycine betaine was greater in Giza 88 than in Giza 70 in both the roots and stems. Treatment with IBA retarded the accumulation of proline and glycine betaine in most cases, except in the stems of Giza 70, where the proline content increased markedly at the highest salinity level.

There was a significant increase in the concentration of Na^+, while the K^+ and Ca^{2+} contents were markedly decreased (Fig. 6). The K^+ content was higher in Giza 88 than in Giza 70. Consequently, the K^+/Na^+ ratio exhibited a decrease under salinity stress. The application of IBA markedly retarded the accumulation of Na^+ and partially alleviated the inhibitory effect of salinity on the K^+ and Ca^{2+} contents and on the K^+/Na^+ ratio in the two cotton cultivars as compared with the values for untreated plants.

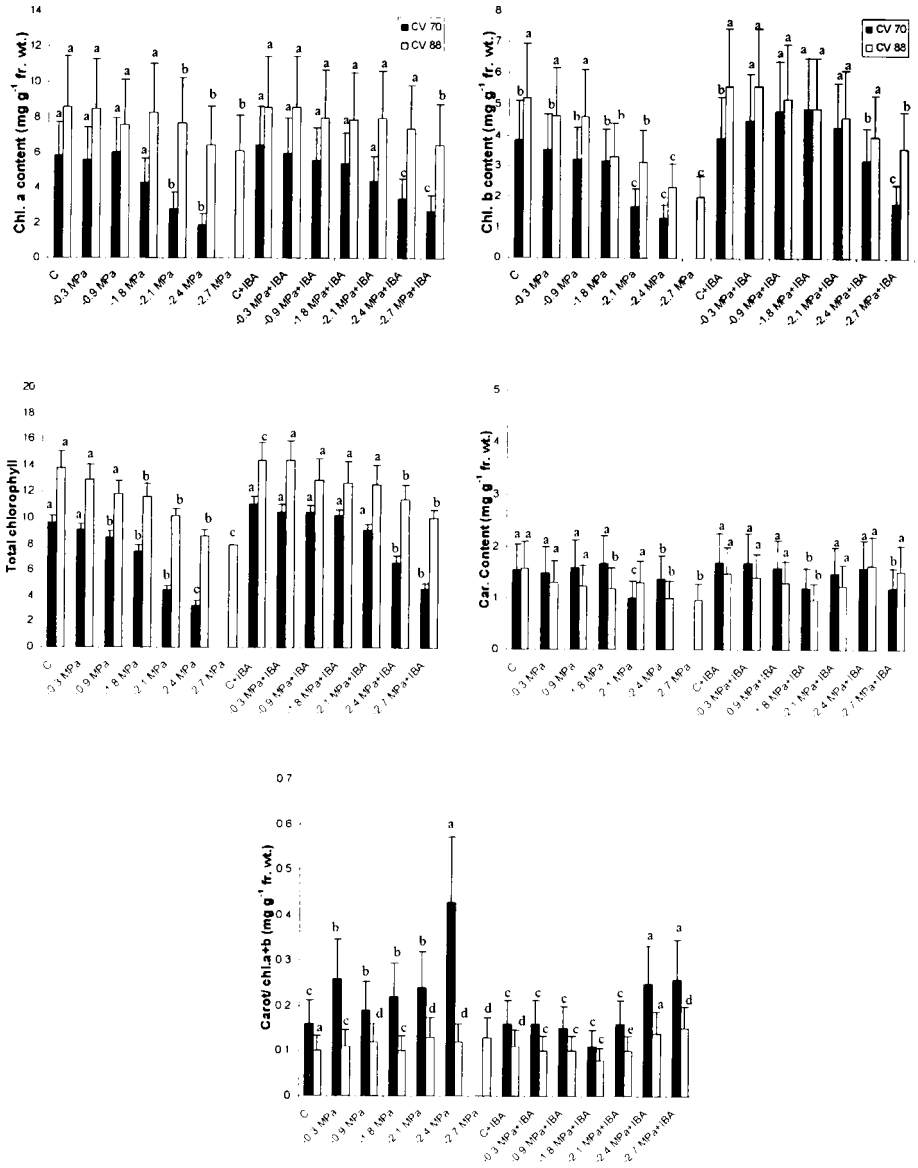

Fig. 2. Effect of salinity and IBA treatment on the content of chlorophyll a, b and carotenoids and the carotenoids/(chl. a + b) ratio of cotton cultivars Giza 70 and Giza 88. Data are presented as means ± SE (n=3). Different letters above the bars indicate significant differences at $p<0.05$ according to Duncan's new multiple range test

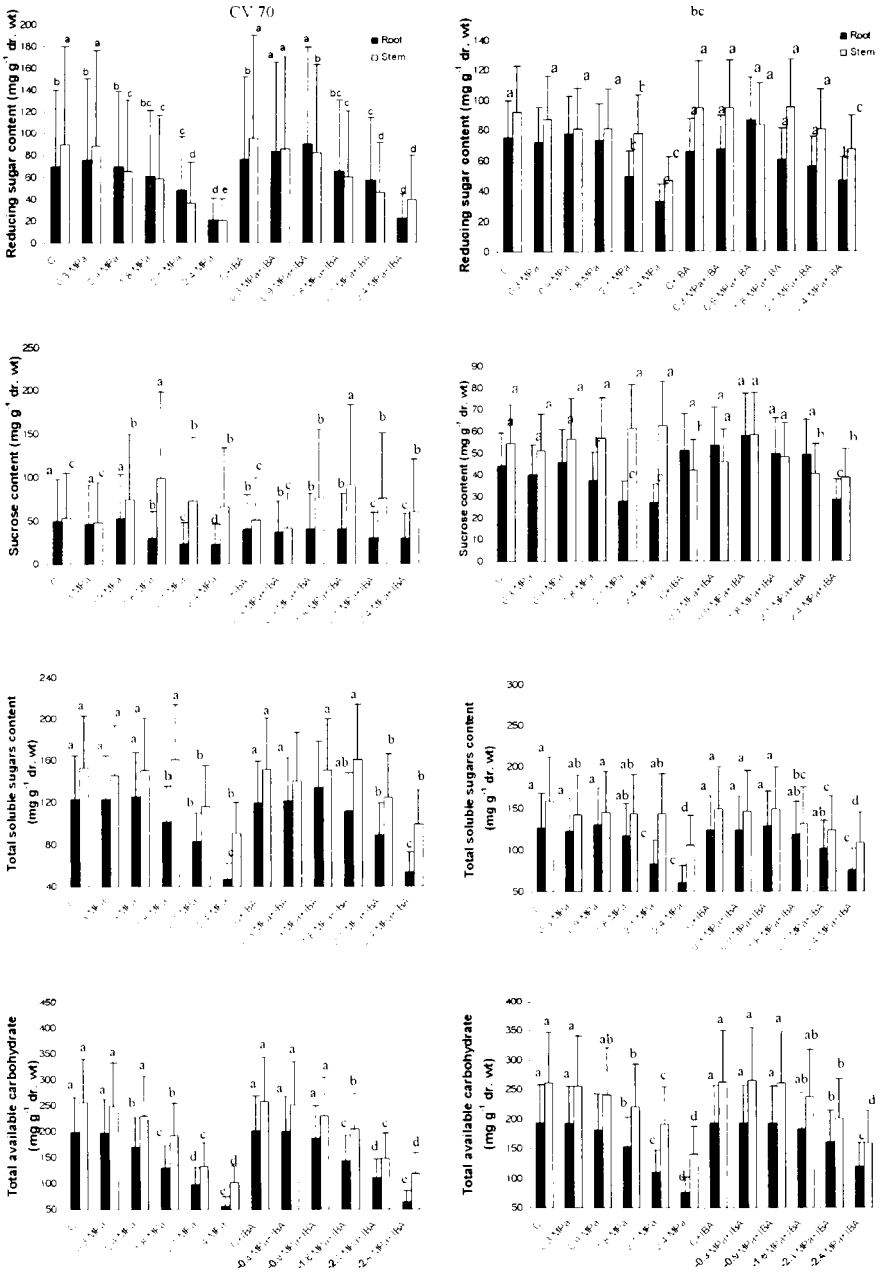

Fig. 3. Effect of salinity and IBA treatment on the carbohydrate content (reducing sugar, sucrose, total soluble sugars and total available carbohydrate) (mg g^{-1} dry matter) in the roots and stems of cotton cultivars Giza 70 and Giza 88. Data are presented as means ± SE (n=3). Different letters above the bars indicate significant differences at $p<0.05$ according to Duncan's new multiple range test

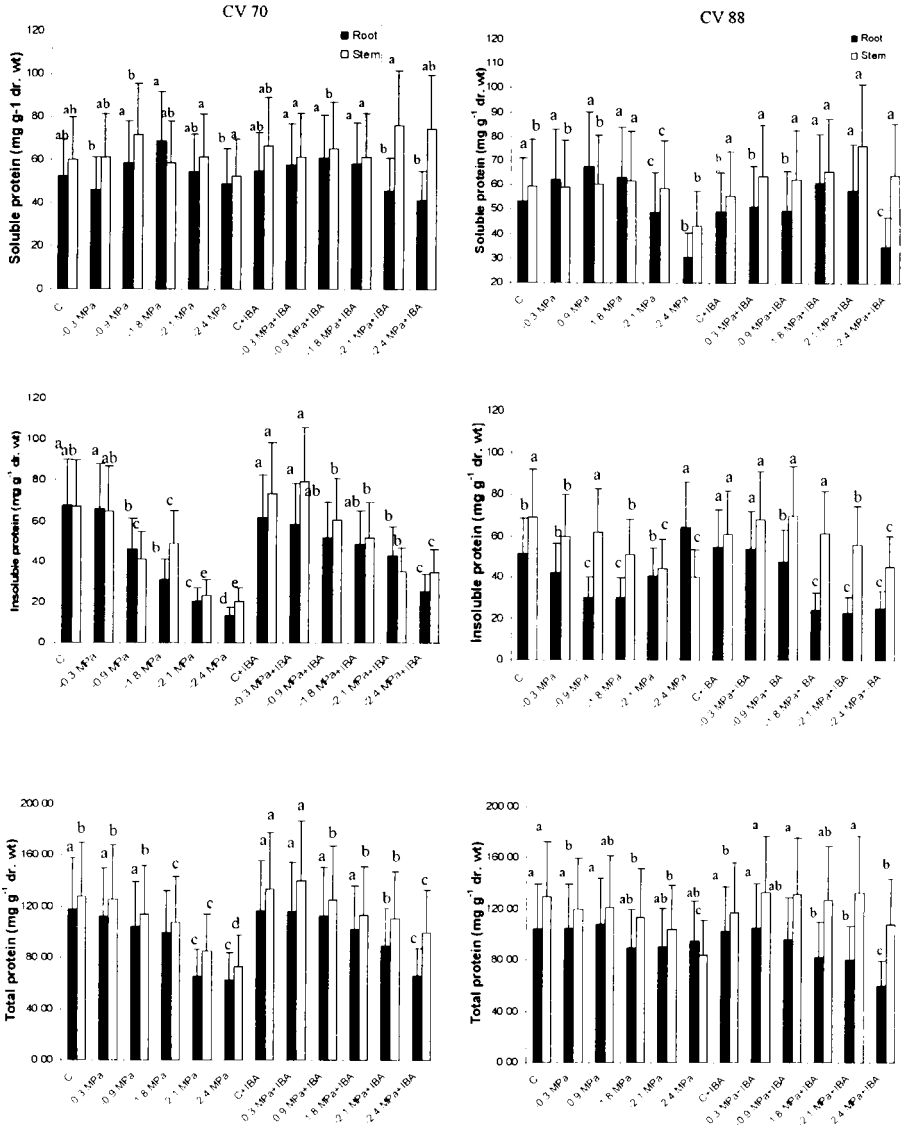

Fig. 4. Effect of salinity and IBA treatment on soluble, insoluble and total protein content (mg g^{-1} dry matter) in the roots and stems of cotton cultivars Giza 70 and Giza 88. Data are presented as means ± SE (n=3). Different letters above the bars indicate significant differences at $p<0.05$ according to Duncan's new multiple range test

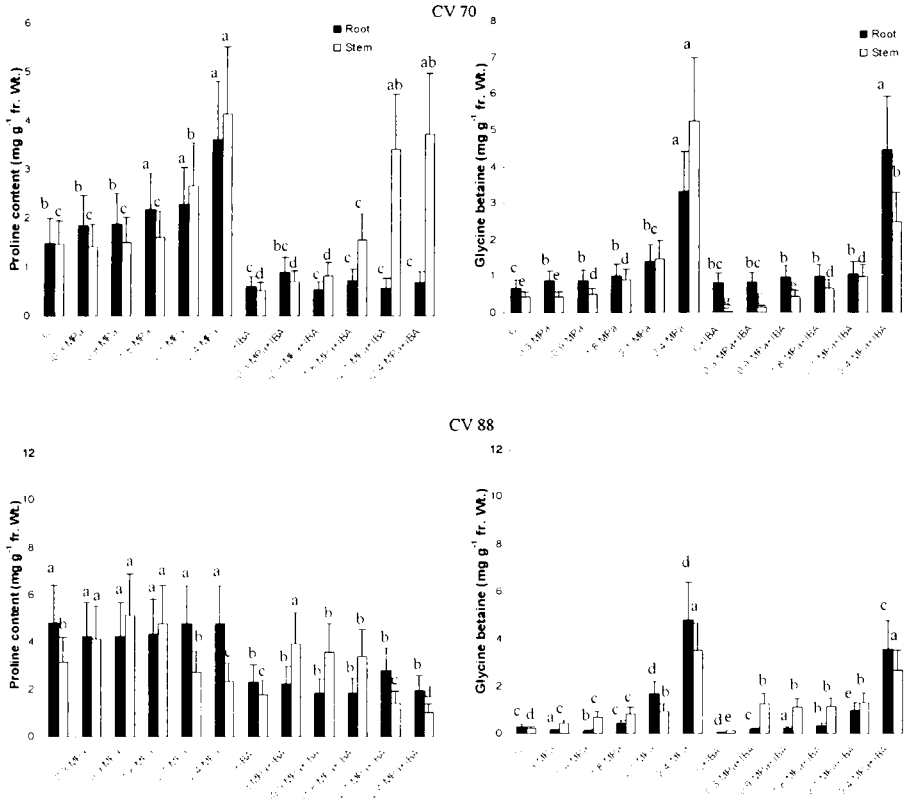

Fig. 5. Effect of salinity and IBA treatment on the proline and glycine betaine (mg g^{-1} fresh matter) in the roots and stems of cotton cultivars Giza 70 and Giza 88. Data are presented as means ± SE (n=3). Different letters above the bars indicate significant differences at $p<0.05$ according to Duncan's new multiple range test

There were pronounced differences in the electrophoretic pattern of the proteins in both cotton cultivars under salinity stress and IBA treatment (Fig. 7). The control samples (lanes 1, 5) showed the presence of 17 peptides with molecular masses between 12.5 and 84 kDa. The major peptides (60, 32, 28 and 22 kDa) had high band intensity. IBA treatment on Giza 70 (lane 2) resulted in the disappearance of the 43 kDa peptide band, while another peptide with 18 kDa appeared with high band intensity. In Giza 88 treated with IBA (lane 6), one new peptide with a molecular mass of 40 kDa appeared on the gel, while other peptides (60, 36 and 22 kDa) were greatly intensified. In the case of Giza 70 (roots + stems) salt treatment resulted in the disappearance of the 40, 26 and 16 kDa proteins (lane 3). The interaction between salinity and IBA treatment in Giza 70 (roots+stems) resulted in the appearance of 12 peptides. Those with

molecular masses of 50, 48, 32, 22 and 12 kDa disappeared, while other peptides with molecular masses of 60, 40, 28 and 18 kDa could only be faintly detected on the gel as compared with the control (lane 4). Salt treatment to Giza 88 resulted in the appearance of one peptide band (43 kDa) with low band intensity (lane 7), while after salt treatment combined with IBA this peptide appeared with high band intensity (lane 8).

Discussion

Salinity is a major environmental factor that limits plant growth and crop productivity (Asish and Anath, 2005), and different crops may have varying salt tolerance mechanisms. The cotton cultivars Giza 70 and Giza 88 tolerated salinity up to the level of –2.4 MPa and –2.7 MPa, respectively. The inhibitory effect of salinity on dry matter and tolerance index was more pronounced in Giza 70 than in Giza 88, which was only slightly affected by salt stress, and recovered quickly after IBA treatment. In contrast, the growth parameters of the salt-sensitive cultivar Giza 70 decreased more drastically as the result of NaCl stress, and did not improve adequately after spraying with IBA. The reduction in growth could be attributed to a reduction in cell division and/or cell enlargement (Hopkins, 1999; Debez et al., 2006).

Spraying the shoots with IBA not only improved the growth of salt-treated Giza 70, but also increased the survival of this cultivar up to –2.7 MPa NaCl. IBA counteracted the inhibitory effect of salinity on the dry matter yield and tolerance index of cotton cultivars. This could be due to the increased flux of water, the decrease in water potential between the plant and soil and the increase in photosynthetic green pigments, leading to a considerable increase in total carbohydrates and protein (Figs. 3 and 4) and improved growth.

Increasing salinity levels in the growth medium of the cotton plants resulted in a significant decrease in the chl.a and chl.b contents. This was accompanied by a significant increase in the carot./(chl.a+chl.b) ratio, particularly in Giza 70 after salinity stress (Fig. 2), indicating the role of carotenoids in the photoprotection of photosynthetic pigments under stress conditions. Treatment with IBA alleviated the inhibitory effect of salinity stress to some extent, especially in Giza 70, suggesting that IBA induced the biosynthesis of photosynthetic pigments, or retarded their degradation. Azooz et al. (2004) reported that IAA ameliorated the inhibitory effect of salinity on the growth of sorghum cultivars, increasing the dry matter, tolerance index and green area. The role of growth substances in overcoming the effects of salinity on growth may be due to changes in endogenous growth regulators, which affect plant water flow (Haroun, 2002).

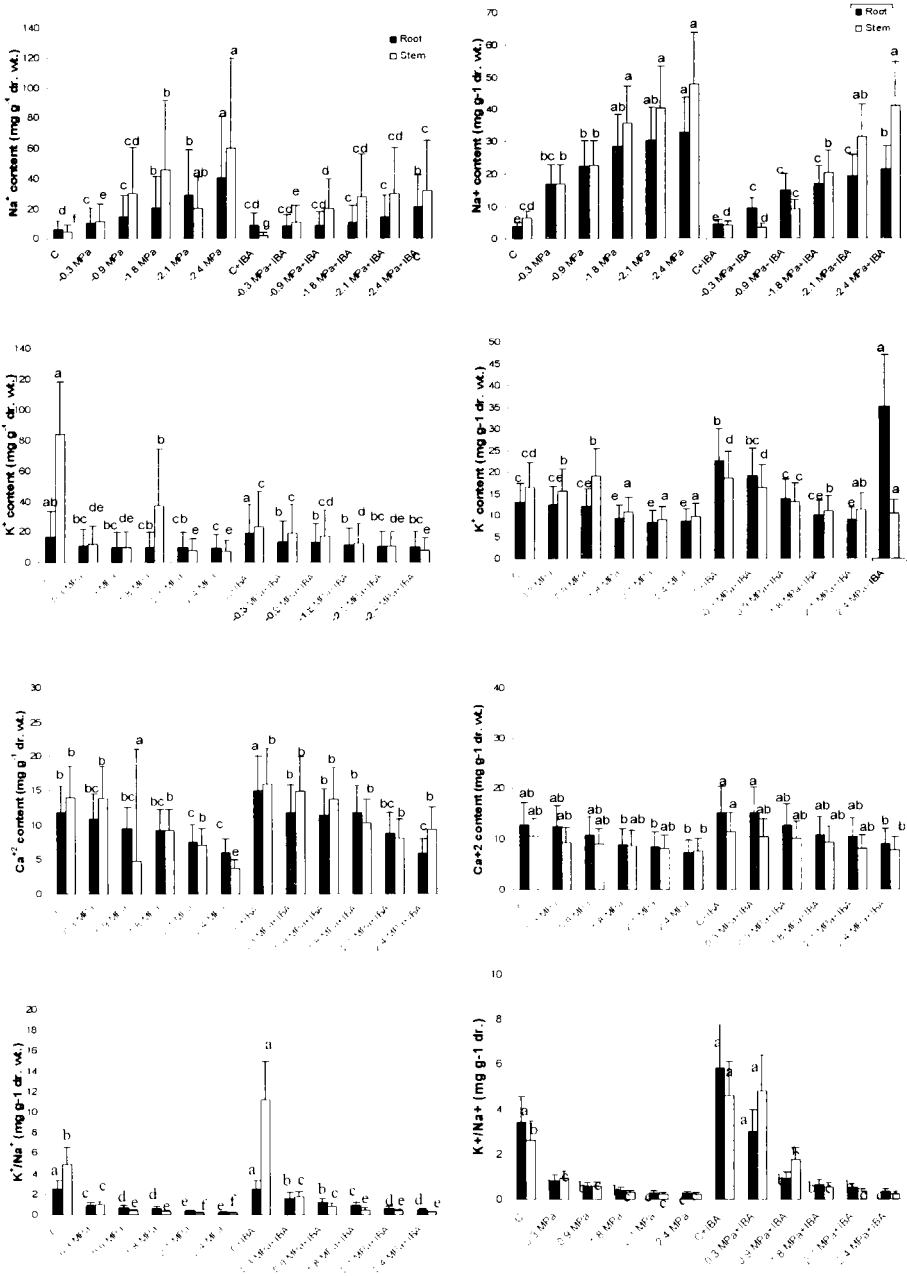

Fig. 6. Effect of salinity and IBA treatment on the Na^+, K^+ and Ca^{2+} contents (mg g^{-1} dry matter) and the K^+/Na^+ ratios of cotton cultivars Giza 70 and Giza 88. Data are presented as means ± SE (n=3). Different letters above the bar indicate significant differences at $p<0.05$ according to Duncan's new multiple range test

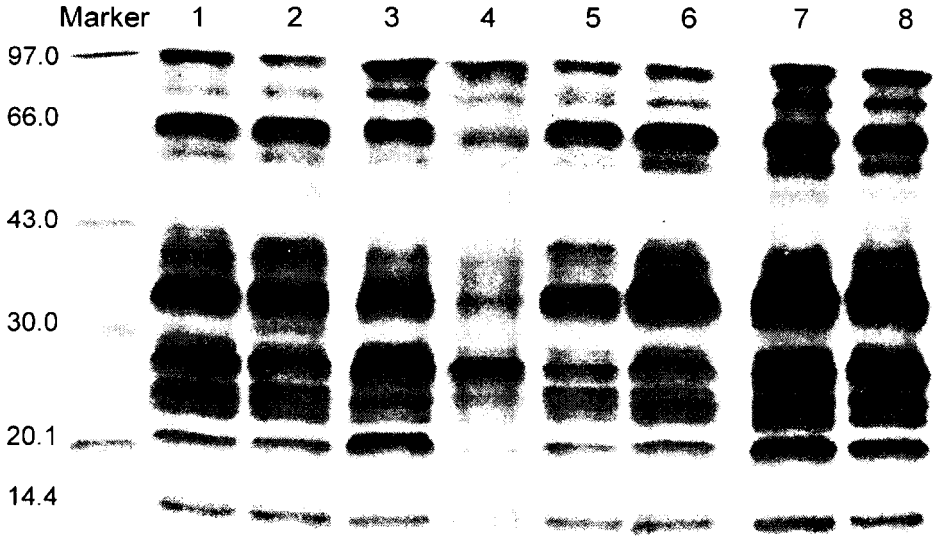

Fig. 7. SDS-PAGE (12.5%T) of the protein in 45-day-old cotton cultivars (roots + stems) in response to salt stress and IBA treatment. 1: Control cultivar Giza 70; 2: Control cultivar Giza 70 + IBA; 3: Salt-stressed (–2.4 MPa) Giza 70; 4: Salt-stressed (–2.4 MPa) Giza 70+IBA; 5 Control cultivar Giza 88: 6: Control cultivar Giza 88 + IBA; =: Salt-stressed (–2.4 MPa) Giza 88; 8: Salt-stressed (–2.4 MPa) Giza 88 + IBA

Carbohydrate accumulation in plants is a well-known means of osmotic adjustment under salt stress (Cheeseman, 1988). In the present work, the content of reducing sugars was higher in the salt-tolerant cultivar Giza 88 than in the salt-sensitive one, indicating better osmotic adjustment ability. Although the TAC contents of the roots and stem of both cultivars were significantly decreased by increasing NaCl concentrations, the TSS/TAC ratios were greatly increased, indicating the role of soluble sugars in osmoregulation and cell turgidity. Sucrose significantly increased in the stems of both cotton cultivars (Fig. 3), thus adjusting the cellular water potential and protecting the plasma membrane from photooxidative damage (Hu et al., 2000).

The total protein content in the roots and stems of both cultivars significantly decreased in response to salinity stress, while the soluble protein content increased significantly in the stem up to a level of –0.9 MPa and to an insignificant extent in the roots up to –2.1 MPa. At higher levels a decrease was recorded in Giza 70. The decrease in the insoluble and total protein in the roots and stems of both cotton cultivars may be due to the suppression of protein synthesis or to greater protein degradation, resulting in a decrease in growth. Mohammed (2007) reported that sugar accumulation and its distribution in different parts of the plant could be a valid trait to discriminate genotypes with different tolerance to saline and osmotic stresses.

Spraying salinized plants with IBA was found to be effective in increasing the carbohydrate and protein contents compared with unsprayed plants. This pronounced accumulation of carbohydrates and proteins may be attributed to the increase in photosynthetic pigments and consequently in plant productivity and dry matter production. The stimulation of protein synthesis in hormone-treated cotton plants may be related to the promotion of mRNA synthesis and polyribosome formation (Romanov, 1990) and to the phosphorylation of ribosomal protein (Yakouleva et al., 1992). It was reported that the increased protein content in response to phytohormones might be due to the increased formation of endoplasmic reticulum, which provides an appropriate medium for the synthesis of polyribosomes and mRNA.

The proline content in the roots and stems of Giza 70 increased significantly with increasing salinity levels in the soil, while in the stem of Giza 88 there was only a significant accumulation of proline up to the −1.8 MPa NaCl level, above which it decreased gradually. These differences may be caused by phenotypic variation between the cotton cultivars. Ueda et al. (2007) reported that salt tolerance is associated with the capacity of species to accumulate proline, which acts as an intracellular osmoticum, thus being involved in osmoregulation. There was a negative correlation between growth parameters and proline content in different organs of cotton cultivars. Proline accumulation due to salt stress was found to be correlated with growth inhibition in rice seedlings and Brassica callus (Perez-Alfocea et al., 1994). Lutts et al. (1996) reported that the rapid accumulation of proline content in plant organs and tissues is one of the most noticeable metabolic consequences of salinity stress.

There was a significant increase in glycine betaine in the roots and stems of both cultivars with increasing salinity levels (Fig. 5), though more was accumulated in Giza 88 than in Giza 70. Glycine betaine may act as a non-toxic osmoticum, preferentially located in the cytoplasm or chloroplast, and may act as an enzyme protectant as well as increasing the ability of cells to retain water without disturbing normal cellular functions (Yancey et al., 1982; Sakamoto and Murata, 2000). It was reported that betaine efficiently protects various components of the photosynthetic machinery, such as ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and the oxygen-evolving phophosystem II (PSII) complex, from salt-induced inactivation and dissociation into subunits (Papageorgiou and Murata, 1995). The osmoprotectant glycine betaine has also been suggested to act as a chaperone, as well as potentially reducing lipid peroxidation and protecting mitochondrial electron transport reactions (Chen and Murata, 2002).

Treatment with IBA retarded the accumulation of proline and glycine betaine in most cases, except in the stems of Giza 70, where the proline content increased significantly at the highest salinity level. This was accompanied by the partial alleviation of the inhibitory effect of salt stress on growth and some related metabolic processes in the two tested cotton cultivars. Azooz et al. (2004)

reported that spraying sorghum cultivars with IAA resulted in a pronounced increase in total free amino acids and retarded the accumulation of proline.

The differences in survival and growth were mirrored by varietal differences in the absorption and accumulation of Na^+ and K^+. Giza 70 seemed to accumulate more Na^+ and less K^+ and Ca^{2+}, and had a low K^+/Na^+ ratio compared to Giza 88 (Fig. 6). The better NaCl tolerance in the latter was associated with a better K^+ supply, resulting in a higher K^+/Na^+ ratio. This again confirmed the salt tolerance of Giza 88. Ghars et al. (2008) reported that the salt tolerance of *Arabidopsis thaliana* and *Thellungiella halophila* may be partially linked to the ability of the plants to control Na^+ influx and to ensure appropriate K^+ nutrition, but is not linked to proline accumulation. Therefore, the high ratio of K^+/Na^+ in Giza 88 may reflect its tolerance to sodium chloride salinity.

Treatment with IBA induced a significant reduction in the absorption and accumulation of Na^+, but increased the K^+ and Ca^{2+} contents and K^+/Na^+ ratio in both cultivars. IBA treatment resulted in an improvement in K^+/Na^+, which might be associated with the reduction of Na^+ accumulation in Giza 70 (salt-sensitive cultivar), while in the more salt-tolerant cultivar Giza 88 it may be associated with the enhancement of K^+ absorption and translocation.

This suggested that the exogenous application of IBA could play an important role in osmoregulation, increasing the efficiency of water utilization under stress conditions and thus increasing the salt tolerance of the experimental plants, while also maintaining adequate levels of these ions to enhance the metabolic processes of the plants. The use of the K^+/Na^+ ratio as an index of salt tolerance is still problematical and could be different for different plants, different cultivars or even for different organs of the same plants.

The influence of phytohormones on the mechanism of ion uptake may be related to its effect on membrane permeability and the rate of ion entry through the membrane or to the enhancement of translocation to the shoots (Haroun, 2002).

There were pronounced differences in the electrophoretic patterns of proteins in both cotton cultivars under salinity stress and IBA treatment (Fig. 7). The control samples (lanes 1, 5) showed the presence of 17 peptides with molecular masses ranging between 12.5 and 84 kDa. The disappearance of the 43 kDa peptide band and the appearance of an 18 kDa peptide after IBA treatment in Giza 70 (lane 2) may be due to the regulation and expression of numerous auxin-responsive genes (Ljung et al., 2002). The disappearance of the 40, 26 and 16 kDa bands in salt-treated Giza 70 (roots + stems) (lane 3) may be the result of high NaCl treatment, as these peptides are salt-sensitive and had a possible role in salt adaptation. Similarly, Parida et al. (2005) reported that a low molecular weight protein (23 kDa) disappeared from the cytosol of cells in the leaves of mangrove plants subjected to salinity stress. The replacement of five peptides by four new peptides in salinized plants of Giza 70 (roots + stems) after IBA treatment suggested that this cultivar favoured the degradation of salt-induced proteins under hormonal treatment. Munoz et al. (1997) reported the

disappearance of a 60-kDa polypeptide in response to salinity in *Prosopsis*. In wheat, the content of a 26-kDa protein increased in NaCl-treated plants, stimulation being more pronounced in the roots than in the shoots, while the contents of 13- and 20-kDa proteins decreased and a 24-kDa protein disappeared after NaCl treatment (Elshintinawy and Elshourbagy, 2001). Tammam (2001) recorded the synthesis of 36 and 32 kDa polypeptides in response to IBA treatment in drought-stressed broad bean lines.

Similarly, a 43-kDa peptide band with low band intensity appeared in salt-treated Giza 88 (lane 7). This peptide was also synthesized when salinized Giza 88 plants were treated with IBA, so this peptide appears to be important in mediating the activation of downstream effectors conferring salinity, possibly leading to an increase in Ca^{2+}, in addition to the activation of other pathways, as reported by Tuteja (2007).

Conclusions

Treatment with IBA alleviated the inhibitory effect of salinity on the dry matter yield and tolerance index of cotton cultivars and increased the growth parameters. It also mitigated the inhibitory effect of salinity on chlorophyll biosynthesis and increased the carot./(chl.a+chl.b) ratio. There was a pronounced accumulation of carbohydrate and proteins compared with unsprayed plants. IBA resulted in a significant accumulation of glycine betaine and retarded the accumulation of proline. There was a significant reduction in the accumulation of Na^+ and a high uptake of K^+, resulting in a high K^+/Na^+ ratio, especially in Giza 88. Therefore, the synthesis and degradation of specific polypeptides in cotton plants under salinity stress indicated that salt stress and phytohormones (IBA) modified the expression of a gene or genes coding for adaptive or defensive proteins.

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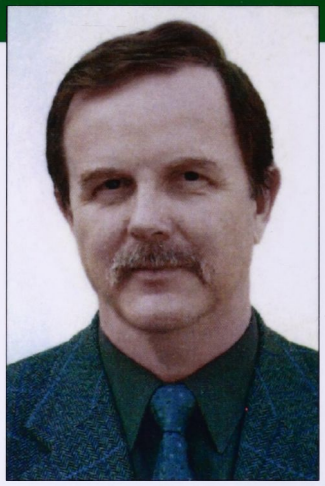

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